Drug modulators of B cell signaling pathways and Epstein-Barr virus lytic activation

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

October 2017

Abstract

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that establishes a latency reservoir in B cells. EBV is the causative agent for several epithelial and B cell cancers mostly in immunocompromised patients. In order to maintain a reservoir of EBV over an infected persons lifetime, EBV must periodically reactivate from latency and enter lytic replication to produce infectious virions, which go on to infect new cells. This can happen spontaneously or by stimulus, such as activating the B cell receptor (BCR) pathway. While the importance of BCR signaling in relation to EBV activation has not been described *in-vivo,* B cell receptor signaling may have an impact on EBV activation in patients. Some B cell malignancies are treated with drugs that inhibit the BCR pathway. Thus it is conceivable that these drugs might affect the viral reservoir maintained by basal B cell receptor signaling.

Here, we show that a group of BCR signaling pathway kinase inihibitors used to treat hematologic malignancies, ibrutinib, idelalisib, and dasatinib, block BCR-mediated EBV lytic induction at clinically relevant doses. siRNA experiments where the targets of these drugs were depleted resulted in the inability of these drugs to block BCR-mediated EBV induction. We found that these drugs specifically block phosphorylation of their targets in the presence of BCR stimulation. Other lytic inducers were tested in the presence of these drugs, and none of the drugs were able to block EBV induction by the other inducers. Thus we believe that blocking BCR-mediated lytic induction by ibrutinib, idelalisib and dasatinib is specific and clinically relevant.

In this work, we also confirmed that the immunosuppressive drugs cyclosporine and tacrolimus inhibit BCR-mediated lytic induction, while rapamycin does not. Rapamycin targets mTOR, which is downstream but still part of the BCR pathway. Treatment of cells with torin2, which inhibits both mTORC1 and mTORC2 prior to EBV induction via the BCR pathway shows that mTORC2 contributes to BCR-mediated lytic induction and that FKBP12 binding alone is not adequate to block activation.

Additionally, we show that BCR signaling can activate EBV in freshly isolated B cells from peripheral blood mononuclear cells (PBMC) and that this activation can be inhibited by ibrutinib or idelalisib.

Taken together, this work shows that ibrutinib, idelalisib and dasatinib block BCR-mediated lytic induction in a specific manner and at clinically relevant doses. While rapamycin does not block BCR-mediated lytic induction on its own, it was determined that mTORC2 plays a role in the BCR pathway and contributes to BCR-mediated lytic activation of EBV. We found that we were able to replicate this system in patient-derived PBMCs, thus suggesting that this system may be relevant in humans and could play a role in future studies on EBV maintenance in the context of long-term treatment by BCR pathway inhibitors.

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Dr. James Stivers

Acknowledgements

Those who have supported me throughout the years have made my thesis dissertation possible. First and foremost, my advisor, Dr. Richard Ambinder, has been a friend and a mentor beyond anything I could have expected. He has been patient, understanding and has allowed me to make mistakes and learn lessons that will guide me for the rest of my life. Rich's knowledge in methodology, scientific thinking and his field of study are a wealth that any student would be lucky to draw upon. His unbridled enthusiasm for science and education has instilled in me a love for science that far surpasses what I previously imagined. Rich has made it possible for me to grow as a scientist out of the lab as well. He has exposed me to international conferences where I was able to present my work to experts in the field and receive feedback that has helped me grow as scientist. My thesis committee has also been a great support during my time as a graduate student. Dr. Prashant Desai has been extraordinarily helpful in lab techniques and methods as well as in broad scientific knowledge. He is masterfully skilled in the lab and is a resource that any student would be lucky to have. Dr. Diane Hayward has spent hours talking with me about the scientific field and experimental design, and these discussions have given me insight into science far beyond my experience. Dr. James Stivers, in whose lab I did my first rotation, has been an excellent resource for experimental discussion and career advice and I am far better off having had these discussions with him.

Without a doubt, one of the greatest sources of comfort and friendship has come from my lab mate Jaeyeun Lee. She is the hardest working, most

intelligent and most thoughtful graduate student I have come across. Our friendship has kept me motivated and I count her as someone who will remain in my life forever. Finally, my parents' unconditional love and support during my time in graduate school has enabled me to focus on my training exclusively, and has proved instrumental in helping me to succeed.

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Introduction

Epstein-Barr Virus

Epstein-Barr virus (EBV), otherwise known as humanherpesvirus 4 (HHV-4), is one of eight human herpesviruses. EBV infects most adults worldwide, and an EBV reservoir is present in an individual for life after initial infection (1). EBV plays a role in numerous diseases, such as infectious mononucleosis and several forms of cancer, both epithelial (nasopharyngeal carcinoma, gastric cancer) and B cell-related (Hodgkin's lymphoma, Burkitt's lymphoma) (2-4). EBV infection is usually symptom-free when contracted while young, but if contracted during teenage or later years, infectious mononucleosis typically presents (5). In immunocompromised patients, such as those suffering from AIDS or having recently undergone transplantation and receiving immumosuppressive drugs, EBV can transform cells and result in cancer (6). Such cancers often regress when treatment by immunosuppressive drugs is withdrawn.

EBV can exist in either a latent or a lytic replication state. Latent replication results in viral genome replication but no virion production, whereas lytic replication results in infectious virion production and subsequent infection of new cells (7).

Virion structure and genome

EBV infection can result in newly produced infectious EBV virions. The viral DNA is surrounded by a nucoelcapsid and tegument. The EBV genome is ~173,000 bases and encodes for approximately 80 protein gene products (8). Finally, a lipid envelope which is embedded with glycoproteins, which serve as a

membrane and the glycoproteins essential for EBV attachment and subsequent infection of cells and viral tropism (9).

Viral entry into cells

EBV infects primarily B and epithelial cells. Four glycoproteins – gB, the gL/gH heterodimer, and gp42 – are required and mediate EBV entry into B cells (10). The gL/gH heterodimer and gp42 form a three-part complex, which is necessary for B cell fusion. gH and gL form heterodimer complexes, which mediate EBV entry into epithelial cells, along with gp350 and an additional heterodimer complex consisting of BMRF2 and BDLF2 (11-15). In B cells, human leukocyte antigen (HLA) class II molecules interact with and degrade three-part complexes in the endoplasmic reticulum, thus EBV produced in B cells contains the heterodimer complex composed of gH and gL, but not the gL/gH/gp42 three-part complex. HLA class II molecules are not present in epithelial cells, resulting in EBV produced in epithelial cells incorporating more gL/gH/gp42 three-part complexes (16). Therefore, EBV produced in B cells is more efficient at infecting epithelial cells, whereas EBV produced in epithelial cells is more efficient at infecting B cells. Viruses without gp42 can bind B cells, but do not enter cells and are replication defective (17).

The mechanism by which EBV enters B cells differs from the mechanism by which EBV enters epithelial cells. In B cells, the EBV glycoprotein gp350 binds CD21 on the cell surface, and g42 interacts with major histocompatibility complex (MHC) class II molecules (10). This results in fusion between the viral envelope and cell membrane, and EBV enters the cell (18).

Infection of epithelial cells by EBV requires the viral protein BMRF-2 and cellular beta-1 integrins. During infection, the gH/gB complex interacts with cellular integrins (10, 19). Once this occurs, fusion of the viral envelope and cellular membrane is possible. In B and epithelial cells, the viral envelope disassociates after the virus enters a host cell.

Lytic replication

During EBV lytic replication cycle, infectious virions are produced. Lytic replication can occur in both B and epithelial cells. In B cells, lytic replication occurs after a latent phase of replication, whereas in epithelial cells, lytic replication typically occurs directly following infection (20). Lytic replication results in linear viral genome becoming packaged into capsids. This is in contrast to latency, where the viral genome persists in a circularized state as an episome. Lytic genes are expressed in stages classified as immediate-early, early and late. Immediate-early genes tend to act as transactivators, such as Zta (Z, Zebra, BZLF1, Zebra transactivator), enhancing the expression of later genes. Early genes have a wider array of functions, such as DNA replication, metabolism and immune system evasion (blockade of antigen expression). Late lytic genes have mostly structural roles, such as capsid and virion structural components (21). BCRF1, a late lytic gene, helps the virus evade the immune system. EBV buds from cells, and lytic infection often leads to cell death.

Latent replication

Latent replication does not result in virion production. During latency, the viral genome circularizes and is maintained as an episome through tethering to

host cell chromosomes by EBNA1 (22). During latency, only small subsets of EBV genes are expressed, and these subsets can be grouped into latency type I, II and III. The genes expressed and latency type is dependent upon the host cell and stage of infection.

Table 1. EBV Latency genes organized by latency type.

Gene	EBNA1	EBNA2	EBNA3A	EBNA3B	EBNA3C	EBNALP	LMP1	LMP2A	LMP2B	EBER
Type	Protein	Protein	Protein	Protein	Protein	Protein	Protein	Protein	Protein	RNA
I	+	-	-	-	-	-	-	-	-	+
II	+	-	-	-	-	+	+	+	+	+
III	+	+	+	+	+	+	+	+	+	+

B cells display all three latency types. Generally, post-infection, EBV progresses from latency III to II to I. After entering a resting B cell, EBV is in latency III, which transforms the cell into a proliferating blast. Subsequently, the virus halts expression of several latent genes and enters latency II, resulting in the cell differentiating into a memory B cell. Ultimately, EBV enters latency I, expressing only EBNA1, which allows for EBV genome replication and partitioning upon cell division (23). Some B cell lines remain at latency III, such as lymphoblastiod cell lines (LCL) and the Raji cell line. In epithelial cells, EBV is only maintained in latency II. Latent infection of B cells may be necessary for viral persistence, replication and release of virions in saliva (24).

Viral Genes and Functions

EBNA1

EBNA1 is perhaps the most important latent protein. EBNA1 is expressed in all types of latent EBV infection, and is found in all EBV-associated cancers.

EBNA1 exists in the cell as a dimer (25). EBNA1 has a glycine-alanine repeat sequence, which separates the protein into amino- and carboxy-terminal domains. The repeat sequence stabilizes the protein by preventing proteasome degradation as well as impairing antigen processing and MHC class I antigen presentation (26-28). This results in the inhibition of the CD8 cytotoxic T cell response against EBV-positive cells (29, 30). EBNA1 is the only protein expressed during latency type I (31). EBNA1 is involved in gene regulation, viral episome replication and partitioning among daughter cells after cell division (32). This occurs through EBNA1 binding to the origin of viral replication (oriP) (33). EBNA1 also binds to the host cell chromosome (22). These two interactions allow EBNA1 to mediate the aforementioned replication and partitioning of EBV genomes.

EBNA2

EBNA2 is the one of several EBV transactivator proteins. EBNA2 helps regulate latent viral transcription and therefore contributes to the transforming capability of EBV (34, 35). EBNA2 also has a role as an adapter protein, binding to DNA-binding proteins CBF1, also known as JK recombination signal protein (RBP-JK), PU.1 as well as some components of RNA polymerase II (34, 36). EBNA2 has a co-activator, EBNA-LP, which helps EBNA2 activate the LMP1 promoter and LMP1/LMP2B transcriptional regulatory element (37).

EBNA3 family

The EBNA3 family includes EBNA3A, EBNA3B and EBNA3C. Investigations have found that EBNA3C is involved in the regulation of the cell

cycle (38). EBNA3A is also required in regulating cell cycle, and it was found that very specific levels of EBNA3A were necessary for LCL maintenance (39). Conversely, EBNA3B appears to restrain the oncogenic capacity of EBV, similar to a tumor suppressor (40). This combination of oncoproteins and tumor suppressor appears to be hitherto unique to EBV.

EBNA-LP

EBNA-LP is a phosphoprotein, which localizes predominately in the nucleus (41, 42). EBNA-LP is an EBNA2 coactivator and is important for B-cell immortalization. In this role, EBNA-LP aids EBNA2 in its activation of the LMP1 promoter and the LMP1/LMP2B transcriptional regulatory element (37).

LMP1

LMP1 is a membrane protein with six transmembrane spans that is essential for EBV-mediated growth transformation (43). LMP1 regulates its own expression as well as that of several host cell genes (44). LMP1 possesses the most potent transforming activity of the EBV latent proteins, and its expression is associated with many of the characteristics of activation of primary B cells and EBV infections (45). LMP1 is a homologue of tumor necrosis factor (TNF) and can mediate signaling in the nuclear factor-kB (NFkB) pathway, in place of CD40 receptor signaling (44, 46).

LMP2

LMP2 includes LMP2A and LMP2B. LMP2A and LMP2B act to inhibit tyrosine kinase signaling. LMP2A is a transmembrane protein that inhibits normal B-cell signal transduction by mimicking an activated BCR. The N-terminus of

LMP2A associates with Src protein tyrosine kinases and spleen tyrosine kinase (Syk), both of which are involved in BCR signaling (47). LMP2B may inhibit such signaling.

EBER (ncRNAs)

Epstein-Barr virus-encoded small RNAs (EBERs) are small non-coding RNAs (ncRNAs) found in the nucleus of cells infected with EBV. EBERs are the most abundant RNA found in EBV-infected cells, and interact with host proteins to form ribonucleoprotein complexes (48). An exact role for EBERs is not yet known, but they may be involved in cell transformation and oncogenesis.

Reactivation

Once latently infected, cells harboring EBV can switch to a lytic replication phase. Lytic replication includes transcription of a host of viral genes, which are categorized as immediate-early, early and late. This can be achieved in the laboratory with chemical or antibody-based agents, but the process by which this occurs *in-vivo* is not well understood. *In-vitro*, stimulation of the B cell receptor by anti-Ig cross-linking and a variety of chemical stimuli, such as sodium butyrate, phorbol esters, proteasome inhibitors and calcium ionophores can induce EBV to replicate lytically (49-53). Since anti-Ig reactivates cells *in-vitro, in-vivo* reactivation may take place in B cells when antibodies against an antigen present on the cell surface bind the infected cell.

ZTA

ZTA, also known as BZLF1, Zebra and Z, is an immediate-early viral gene of EBV. The Z promoter, Zp, can be bound by a multitude of transcription

factors, and such binding facilitates Zta transcription. Similar to many transcription factors, ZTA has a leucine zipper domain, and exists as a homodimer (54, 55). The expression of ZTA is sufficient to turn on lytic replication, resulting in the production of infectious virions. ZTA binds to oriLyt (lytic origin of replication) on the EBV genome, the BALF2 promoter and interacts with a number of replication proteins (56, 57). ZTA is widely used as an indicator of lytic replication in viral assays. ZTA can bind to Zta response elements (ZREs), such as those contained within the BRLF1 gene and the origin of replication (oriLyt) (58).

RTA

RTA is the product of the BRLF1 gene and functions as a transcriptional activator in both B and epithelial cells (59). Rta contains ZREs, sequences to which ZTA can bind to enhance transcription (58). Rta expression has also been shown to reactivation in epithelial cells, and more recently in B cells. RTA-induced latent disruption and subsequent lytic replication appears to be less potent than ZTA-induced lytic activation. Additionally, transfection of RTA into HH514-16 cells leads to ZTA synthesis and viral DNA replication (21).

Transformation of B cells

EBV has the capability of transforming B cells to divide indefinitely. To do so, B cells, separated from PBMCs are infected with EBV and maintained in culture. LMP1 seems to be the main driver of transformation, while EBNA2 and EBNA3C also play a role (34, 35, 38, 39, 45, 60).

EBV types

Broadly, EBV has two subtypes – type I and type II. The main difference is in the EBNA2 and EBNA3 genes (61). This manifests itself in different transforming capabilities between the two subtypes of EBV. While type I is dominant around much of the world, in Africa, types I and II exist in equally distributed amounts. Restriction enzyme digestion of the viral genome enables distinction between the two types. Differential gene regulation between type I and type II EBV have been shown to account for the weaker B cell transformational capacity of type II EBV versus type I (62).

Role in disease

Infectious mononucleosis, Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and other diseases have been linked to EBV (2-4). While most individuals will maintain EBV with no symptoms throughout their lifetime, immunocompromised patients are at an increased risk for EBV-related malignancies (6).

B cells

The B cell receptor and EBV activation

The B cell receptor is composed of two parts: an immunoglobulin molecule including IgD, IgM, IgA, IgG or IgE and the signal transduction molecule CD79. Upon being bound by an anti-Ig molecule, the BCR signaling pathway is activated and results in a downstream phosphorylation cascade involving proteins such as LYN, SYK, PI3Kδ, PLC-γ, AKT, BTK and ERK (63, 64). This activation results in a calcium flux that can disrupt latently replicating EBV in certain cell lines (65, 66).

There are several drugs used to treat hematologic malignancies that block signaling kinases in the BCR pathway. Idelaliaib blocks PI3Kδ, ibrutinib blocks BTK and dasatinib blocks BTK and LYN as an off-target effect (it is used clinically to inhibit ABL kinase) (67-69). There may be cross-talk within this pathway, resulting in idelalisib blocking phosphorylation of BTK and ibrutinib blocking phosphorylation of PI3Kδ (70).

B cell malignancies and EBV

There are a number of B cell cancers where EBV is involved. These include Burkitt lymphoma (BL), post-transplant lymphoproliferative disorder (PTLD), AIDS lymphoma and immunodeficiency lymphoma.

Burkitt lymphoma

BL is a B lymphocyte malignancy originating in the jaw. BL presents most frequently in children in parts of the world where malaria is endemic. It is hypothesized that malaria infection weakens the hosts immune system, aiding EBV infection in taking hold and persisting, allowing it time to transform cells and result in cancer. The pathogenesis of AIDS-associated BL appears to differ from that of classic BL (71).

Post-transplant lymphoproliferative disorder

PTLD is a malignant proliferation of B-lymphocytes, which carry EBV under latent replication. PTLD arises in immunosuppressed patients who have recently undergone transplant and are under a regimen of immunosuppressives, such as cyclosporine and tacrolimus which block T cell function. Blockade of T cell function and resultant loss of immunosurveillance is thought to allow EBV to

transform cells unchecked. Rapamycin, another immunosuppressive, leads to an increase in T cell function in certain cases (72).

AIDS lymphoma

AIDS lymphoma describes lymphomas present in patients with AIDS. Generally, a pool of AIDS patients has an increased frequency of lymphomas than the general population. This observation is most likely due to a lack of immunosurveillance, parallel to PTLD.

Immunodeficiency lymphoma

Lymphomas can also occur in patients with primary immunodeficiency. Such a case might occur in patients with Ataxia Telangiectasia and Wiskott-Aldrich syndrome (73). Rather than being immunocompromised by immunosuppressive drugs or have an acquired immunodeficiency such as those affected by AIDS, or more mildly, malaria as in BL, these patients have rare, chronic disorders that predispose them to malignancies.

Importance

EBV establishes viral latency in B cells. Activation of the BCR pathway activates EBV in cell lines. This work demonstrates that drugs that inhibit kinases in the BCR signaling pathway inhibit activation of EBV, but do not inhibit several other lytic activation pathways. Immunosuppressant drugs cyclosporine and tacrolimus but not rapamycin also inhibit BCR-mediated EBV activation. Additionally, we show that BCR-activation of lytic infection occurs in freshly isolated B cells from patients and that this activation can be blocked by BCR-inhibitors.

Chapter 1: Characterization of kinase inhibitors on EBV-positive cell lines

SUMMARY

Epstein-Barr virus is a human gammaherpes virus that primarily infects B cells. In an immunocompromised host with such an infection a variety of B cell malignancies have been observed. Several recently FDA-approved kinase inhibitors affect the BCR pathway, and it is possible to disrupt EBV latency by stimulation of this pathway. Thus we reasoned that the use of kinase inhibitors blocking the BCR pathway might have an impact on BCR-mediated EBV reactivation. We found that ibrutinib, which inhibits BTK, idelalisib, which inhibitits PI3Kδ, and dasatinib, which is used to target ABL kinase, but also inhibits LYN all inhibit BCR-mediated EBV reactivation at clinically relevant doses. We also found that siRNA knockdown of BTK, PI3Kδ and LYN resulted in a decrease in lytic induction by BCR pathway stimulation, confirming this pathway as being involved in such activation. Further confirmation of this was achieved by the observation of the inability of ibrutinib, idelalisib and dasatinib being unable to block induction of EBV lytic production by other lytic stimuli such as ionomycin, sodium butyrate and phorbol esters. Finally, we were able to show that in freshly isolated B cells, ibrutinib and idelalisib blocked BCR-mediated EBV activation.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous human gammaherpesvirus infection that is maintained in a fraction of resting memory B cells following primary infection (74-76). The elimination of B cells from blood following treatment with a B cell-targeting monoclonal antibody commonly results in an inability to detect EBV DNA in PBMCs (77). The reservoir of EBV in B cells appears to be necessary to maintain the virus as evidenced by the observation that chronic infection is not established in patients with Bruton agammaglobulinemia who lack B cells (76). While the virus resides mainly in B cells, the character of the B cells in which the virus is harbored affects viral gene epression. *In vitro*, EBV infection of B cells results in immortalized LCLs expressing eight or more latency antigens whereas *in vivo*, in healthy seropositive adults the B cells that harbor viral genomes demonstrate very restricted viral gene expression. The germinal center reaction that occurs in lymphoid tissue is hypothesized to play a key role in down modulating viral gene expression (74). In some B cell tumor lines, BCR signaling is a potent activator of EBV lytic gene expression (78). EBV gene expression, particularly expression of LMP2A and LMP2B influence the BCR signaling pathway (79). Thus B cells harbor EBV and B cell biology is important to the viral life cycle.

Several pharmacologic agents that target B cell signaling pathways have come into clinical use. These include agents used because they target BCR signaling pathways such as ibrutinib, which inhibits BTK, and idelalisib which

inhibits PI3Kδ; and other agents with off-target effects on BCR signaling such as dasatinib, which targets Bcr-Abl kinase but also has effects on LYN.

In this work, we show that some BCR signaling inhibitors profoundly inhibit BCR-driven activation of lytic infection in B cell tumor lines that harbor EBV. Finally, we show for the first time that BCR signaling is an activator of EBV lytic infection in naturally infected patient B cells and that ibrutinib and idelalisib block this activation.

Materials and Methods

Reagents and Antibodies. Antibodies against phospho-AKT (Ser473) (Cat# 9272S), Akt, phospho-BTK (Tyr223) (Cat# 5082P), phospho-ERK (Thr202/Tyr204) (Cat# 9101S), and ERK (Cat# 4695S) were obtained from Cell Signaling Technologies. Antibodies against BTK were obtained from R&D Systems (Cat# MAB5807). Antibodies against phosopho-PI3Kδ (Tyr485) (Cat# sc-130211), PI3Kδ (Cat# sc-7176) and EBV ZTA (Cat# sc-53904) were obtained from Santa Cruz Biotechnology and against β-actin (Cat# A5441) from Sigma-Aldrich. Anti-IgG and anti-IgM were purchased from Sigma-Aldrich (I5260 and I0759, respectively). In all experiments where used, anti-IgG and anti-IgM were used at 10 μg/mL. Secondary anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch. Ibrutinib was purchased from ApexBio. Idelalisib and dasatinib were obtained from MedKoo. Ionomycin was purchased from Sigma-Aldrich.

Cell Lines and Culture. An engineered Akata cell line derivative (BX1 Akata) that carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP) was a gift from L. Hutt-Fletcher (80). BX1 Akata cells were cultured in RPMI 1640 media (Mediatech) with 10% FBS (Mediatech), 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies), 100 mM L-glutamine (Life Technologies), and 500 μg/mL G418 (Life Technologies).

Fluorescence Microscopy. $1.5x10^5$ cells were taken from culture and washed with PBS. Cells were then spun onto microscope slides using a Cytospin centrifuge. Cells were fixed and permeabilized with ice-cold methanol for 15

minutes and blocked in 5% BSA in PBS for 30 minutes at room temperature. ZTA antibodies (Santa Cruz) were diluted 1:50 in 5% BSA in PBS and applied to the cells for 1 hour at room temperature. The attached cells were then washed 3 times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS. Cy3 goat anti-mouse antibodies (Jackson Immunoresearch) were diluted 1:50 in 5% BSA in PBS and incubated with to the cells for 1h at room temperature in the dark. The slides were then washed 3 times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS. The cells were stained with Vectashield mounting media with DAPI (Vector Laboratories). For GFP, BX-1 Akata cells were cultured as above and treated as indicated in the figures. A ZOE Fluorescent Cell Imager (Bio-Rad) was used to image the fixed cells for Cy3 and live cells expressing GFP.

Quantitative PCR. DNA was isolated from cells with the QIAamp DNA isolation kit from Qiagen. Quantitative PCR assay of the Bam W repeat region of the EBV genome was performed for measuring viral load. A BamW probe (Integrated DNA Technologies) (5′ (FAM) CACACACTACACACACCCACCCGTCTC (BH1) 3′) was used in conjunction with SsoAdvance Supermix (Bio-Rad). BamW primers were obtained from Integrated DNA Technologies (fwd 5′ CCCAACACTCCACCACACC 3’ and rev 5′ TCTTAGGAGCTGTCCGAGGG 3′). Copy number was determined by comparing to a serial dilution of the Namalwa cell line. The concentration of primers was 500 nM. The probe concentration was 200 nM. 2 µL DNA at 50 ng/mL was used per reaction. Reaction size was 20 µL. The reaction was set at 95°C for 2 minutes for 1 cycle and then 95°C for 5 seconds and 60°C for 10 seconds for a

total of 40 cycles on a CFX96 real time thermocycler from BioRad. For viral load quantification by qPCR, cells were treated with ibrutinib, idelalisib or dasatinib for 1h prior to treatment with anti-IgG, and DNA was isolated after 48h. For Zta mRNA quantification, cells were treated as above and RNA was isolated after 24h (RNEasy kit from Qiagen). cDNA was then made from the RNA using the iScript reverse synthase kit from BioRad. cDNA volume corresponding to 25 ng total RNA was amplified using the SsoFast Evagreen kit from BioRad on a CFX96 real time thermocycler from BioRad. The cDNA was amplified using a PCR program of 95°C for 30 seconds once and then 95°C for 5 seconds, and 60°C for 5 seconds for 40 cycles. Primers used for Zta were fwd 5'ACATCTGCTTCAACAGGAGG5 3' and rev 5'AGCAGACATTGGTGTTCCAC 3'.

Immunoblots. For phospho-protein immunoblots, cells were serum-starved for three hours and pre-treated with ibrutinib, idelalisib or dasatinib for 1h before treatment by anti-IgG. Protein was isolated ten minutes after treatment by anti-IgG. For ZTA blots, cells were treated with ibrutinib, idelalisib or dasatinib for 1 hour prior to anti-IgG treatment and protein was extracted 24h after anti-IgG treatment. To prepare protein extracts, 1.5 X 10^7 cells were pelleted by centrifugation and washed in PBS. The pellet was resuspended in a 10 mM HEPES, pH 7.9, 10 mM KCl, 100 µM EDTA and 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies) buffer and incubated on ice for 15 min. NP-40 was added to a final concentration of 0.6% and the cells were lysed by brief vortexing. The cytosolic supernatant was separated from the nuclear

pellet by centrifugation at 10,000 rpm for 30s at 4°C. The nuclear pellet was resuspended in a 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA buffer with 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies) followed by rotation for 15 minutes at 4°C. The nuclear supernatant was isolated by centrifugation at 13,000 rpm for 5 minutes at 4°C. Equal amounts of protein per sample were separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes. After probing with primary antibodies, horseradish peroxidase-conjugated antibodies (GE) were applied against the primary antibodies. The immunoblots were imaged using Amersham ECL-Prime chemiluminescent reagent (GE Health). The film used was HyBlot CL autoradiography film (Denville Scientific).

Statistical Analysis. IC_{50} values were calculated using $Y=Y_{min} + (Y_{max}-Y_{min})/(1+10^{((X-LogIC50)))}$ in GraphPad Prism.

Patient specimens. With written informed consent and approval of the Johns Hopkins Hospital Investigational Review Board, blood was collected from two older adult patients with night sweats, fevers and elevated EBV copy number in PBMC. Both had diffuse small lymphadenopathy (<2cm) and biopsies that showed atypical follicular hyperplasia with no evidence of clonality by immunohistochemistry or flow cytometry. Both patients were ultimately treated with rituximab and had only transient clinical responses.

MACS isolation of patient B-cells. PBMCs were isolated from whole blood using the Ficoll gradient method. B cell subsets were isolated by depletion of

magnetically labeled non-B cells from patient PBMCs (B Cell Isolation Kit II, Miltenyi Biotec).

siRNA knockdown. SMARTpool siRNA against BTK (M-003107-01-0005), LYN (L-003153-00-0005) and PIK3CD (L-006775-00-0005) were purchased from Dharmacon and nucleofected into BX1-Akata cells at 30 nM (BTK and LYN) and 300 nM (PIK3CD). Nucleofection was carried out using a Nucleofector II device from Amaxa using Amaxa Cell Line Nucleofector Kit R according to the manufacturer's specifications. Forty-eight hours after the initial nucleofection, the nucleofection was repeated on the same cells. 24 hours after the second nucleofection, the cells were treated with 10 μg/mL anti-IgG. Images were taken and RNA extracted 24 hours after anti-IgG treatment.

Results

Previous investigators demonstrated that anti-Ig induces lytic EBV replication in the Akata Burkitt cell line (81). We studied lytic gene expression and GFP expression in the BX1-Akata cell line, where GFP served as an indicator of lytic replication of EBV (Fig. 1-1 and 1-2). Induction with anti-Ig led to increased viral lytic RNA, protein, viral DNA, and increased GFP expression. Thus in the BX1-Akata cell line, GFP expression parallels other indications of EBV lytic gene expression. We investigated whether or not BCR kinase inhibitors would block BCR-mediated EBV reactivation by treating with kinase inhibitors known to block BCR signaling: ibrutinib, idelalisib or dasatinib. Each of these agents also decreased basal levels of Zta RNA and protein, likely reflecting inhibition of basal BCR signaling, and in combination with anti-Ig, blocked the induction of Zta RNA, ZTA protein, GFP fluorescence, and EBV DNA replication.

To determine the potency of these drugs in blocking of BCR-mediated EBV activation, we performed dose-response experiments on BX1-Akata cells. We calculated the IC_{50} for inhibition of GFP expression (Fig. 1-3). These results parallel values reported in the literature for inhibition of BTK, PI3Kδ and LYN enzymatic activities (82-84). As shown in Fig. 1-4, these agents block anti-Ig induced phosphorylation of downstream B-cell receptor signaling proteins. Thus the pharmacodynamics of inhibition of EBV lytic induction paralleled the inhibition of the expected target pathway, i.e. ibrutinib, idelalisib and dasatinib all inhibited phosphorylation of AKT, ERK and PI3K. Note that clinically dasatinib is used to inhibit ABL kinase and inhibition of LYN is an off-target effect (84). To validate

the drugs specificity on their target genes, we undertook siRNA knockdown experiments against BTK, LYN and PI3K (Fig. 1-5). In each siRNA experiment, anti-IgG mediated increased GFP and Zta expression was blocked relative to control samples.

To further explore the effects ibrutinib and idelalisib on EBV activation, we studied the effects of other EBV lytic inducers: ionomycin, TPA and NaB (Fig. 1-6, 1-7 and 1-8). No inhibition of lytic activation was apparent. At lower doses of TPA and NaB, ibrutinib and idelalisib had no effect on lytic activation (data not shown). Thus inhibition of lytic induction is not a general phenomenon but seems to be specific to BCR-activated lytic induction.

Although many investigators have documented the effects of BCR-signaling on activation of EBV in a variety of cell lines (49, 78, 85), to the best of our knowledge these effects have never been documented directly in B cells from patients. For this investigation, we obtained PBMCs from two patients with high EBV copy number without malignancy. We isolated B-cells and treated with anti-IgM in combination with ibrutinib or idelalisib (Fig. 1-9). We found that BCR stimulation activated EBV lytic replication as shown by increased viral DNA but that this effect was blocked by ibrutinib and idelalisib. Thus BCR signaling can activate EBV replication in non-malignant cells and pharmacologic agents that block the BCR signaling pathway inhibit this activation.

Figure 1-1

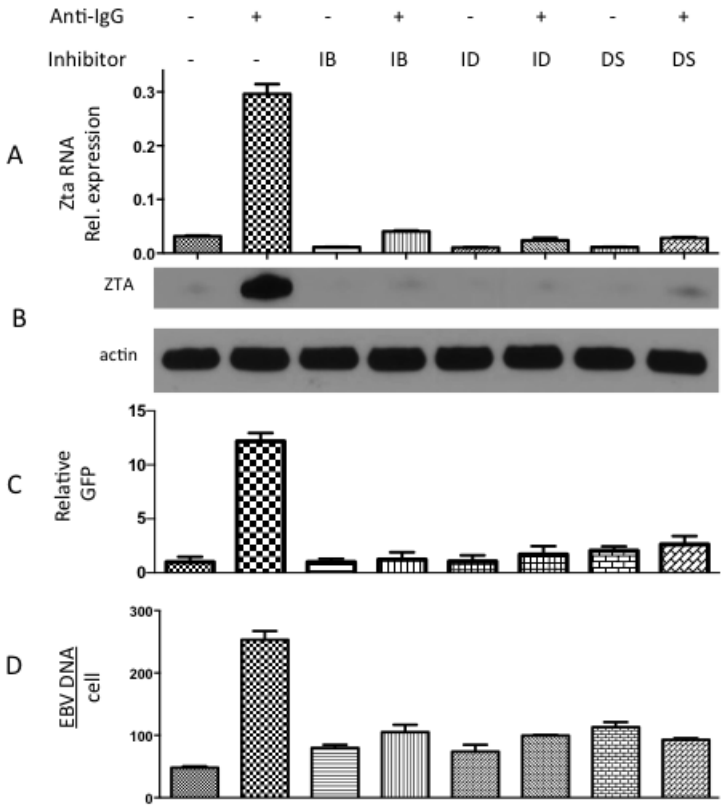

Figure 1-1. Ibrutinib, idelalisib and dasatinib block BCR-mediated EBV activation. BX1-Akata cells were treated with 1 μM ibrutinib, idelalisib or dasatinib for 1 hour, followed by induction with anti-IgG. A. qRT-PCR amplifying Zta cDNA 24 hours after treatment with anti-IgG normalized to GAPDH. B. Immunoblot showing ZTA 24 hours after treatment with anti-IgG. C.GFP-positive cells were quantified and compared with an untreated sample 24 hours after treatment. D. qPCR amplifying the BamW region of the EBV genome 48 hours post-treatment of anti-IgG.

Figure 1-2

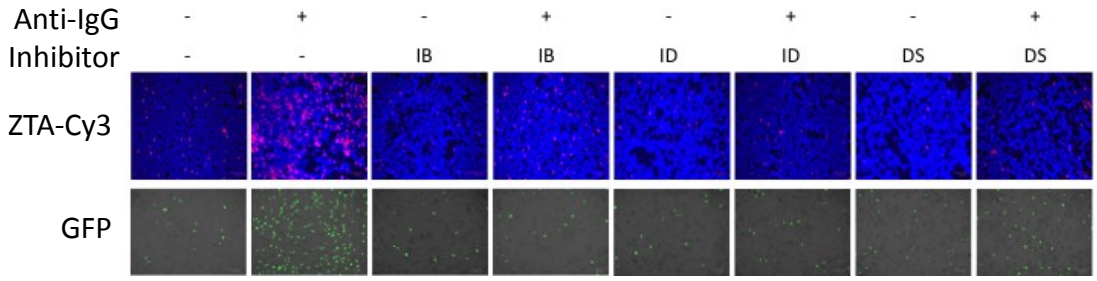

Figure 1-2. Ibrutinib, idelalisib and dasatinib block BCR-mediated EBV activation. (Top panel) Immunofluorescence showing ZTA 24 hours after treatment with anti-IgG. (lower panel) fluorescent cells expressing GFP 24 hours after treatment with anti-IgG.

Figure 1-3.

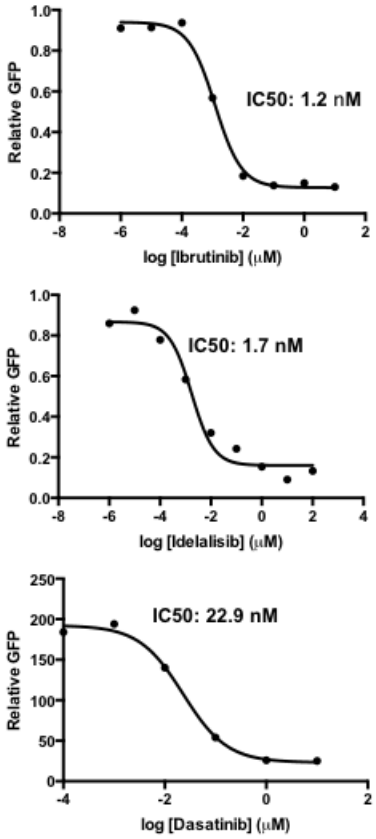

Figure 1-3. Ibrutinib, idelalisib and dasatinib inhibit BCR-mediated EBV activation in a dose-dependent manner. GFP-positive cells were quantified and plotted against drug concentration 24 hours after anti-IgG treatment. Graphs are representative of three or more independent experiments.

Figure 1-4.

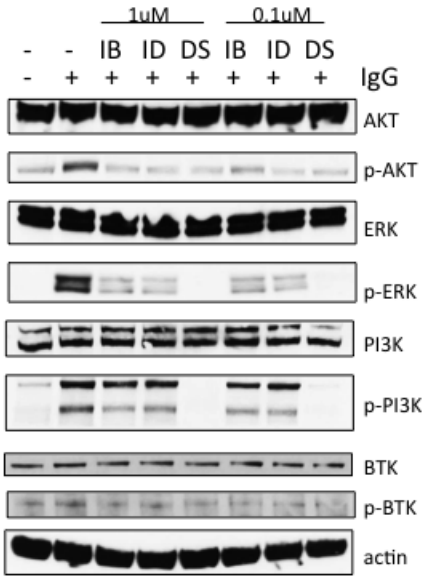

Figure 1-4. Ibrutinib, idelalisib and dasatinib block B cell receptor pathway component phosphorylation. Cells were treated with 1 or 0.1 μM ibrutinib, idelalisib or dasatinib for 1 hour followed by induction with anti-IgG. Protein was isolated 10 minutes after anti-IgG treatment and analyzed by immuno blotting. Blotting is representative of three or more independent experiments.

Figure 1-5.

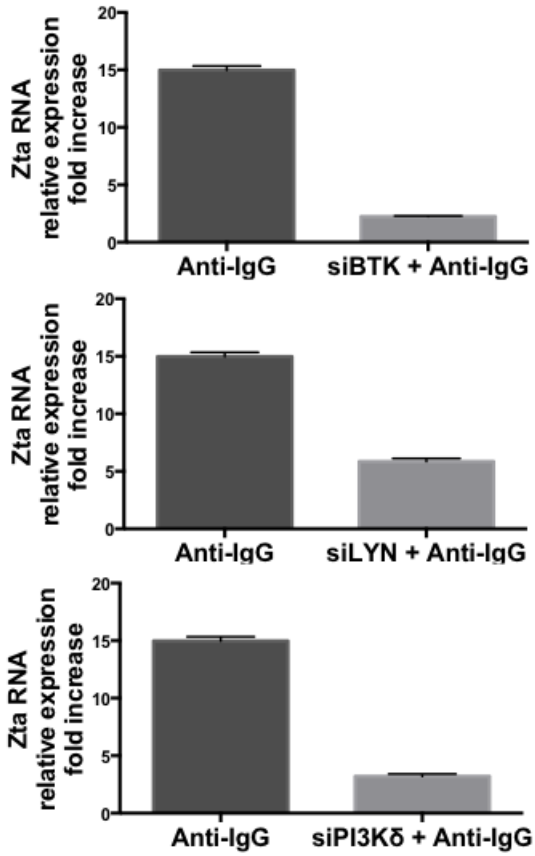

Figure 1-5. siRNA knockdown of drug targets results in decreased ability of B cell receptor pathway stimulation to induce lytic replication of Epstein-Barr virus. BX1-Akata cells were nucleofected with siRNA targeting the indicated transcripts followed by treatment with anti-IgG. qRT-PCR amplifying Zta cDNA 24 hours after treatment with anti-IgG. Zta levels were normalized to GAPDH and compared to a control sample.

Figure 1-6.

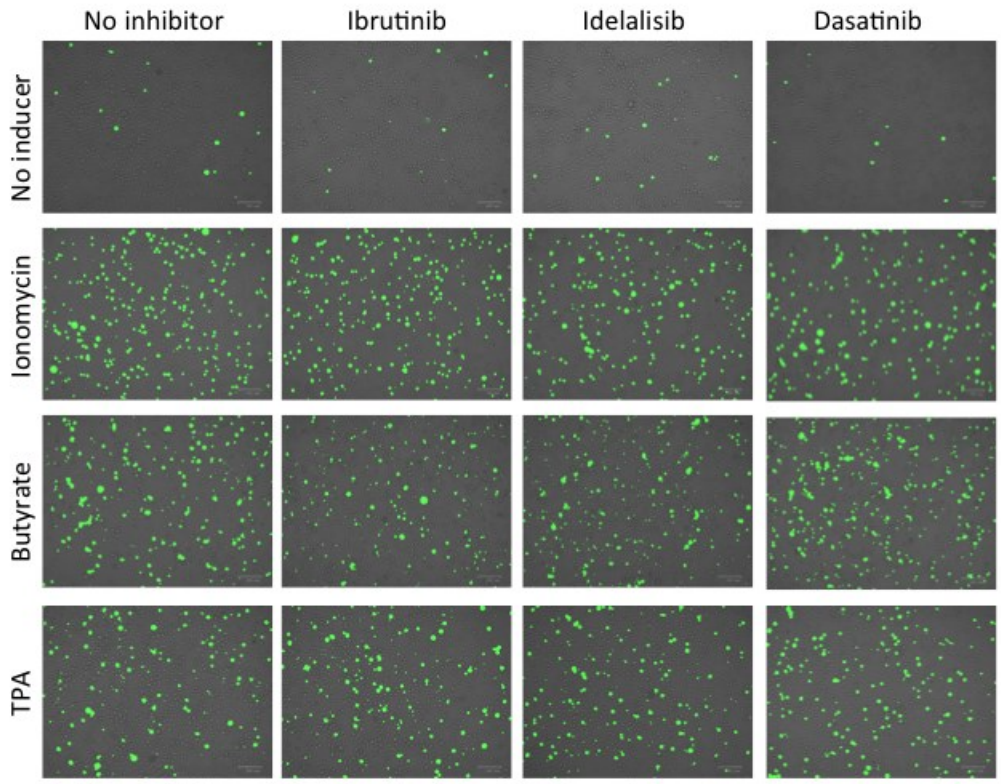

Figure 1-6. - **BCR pathway kinase inhibitors in combination with other inducers.** BX1-Akata cells were pretreated with the indicated kinase inhibitors for 30 minutes and then treated with the indicated lytic inducers. Cells were imaged for GFP 24 hours after treatment with inducers. Cells were treated with 1 μM ionomycin, 20 ng/mL TPA or 3 mM NaB.

Figure 1-7.

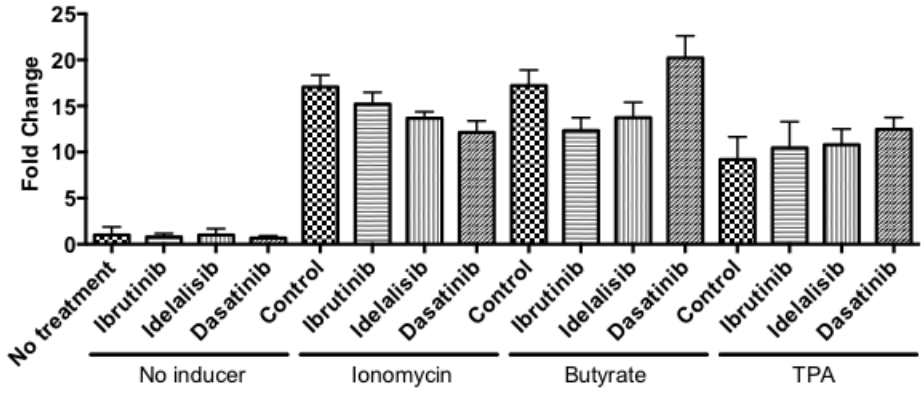

Figure 1-7. Kinase inhibitors with other lytic gene inducers. BX1-Akata cells were pretreated with the indicated kinase inhibitors for 30 minutes and then treated with the indicated lytic inducers. Cells were imaged for GFP 24 hours after treatment with inducers. GFP-positive cells were quantified and compared to an untreated sample. Cells were treated with 1 μM ionomycin, 20 ng/mL TPA or 3 mM NaB.

Figure 1-8.

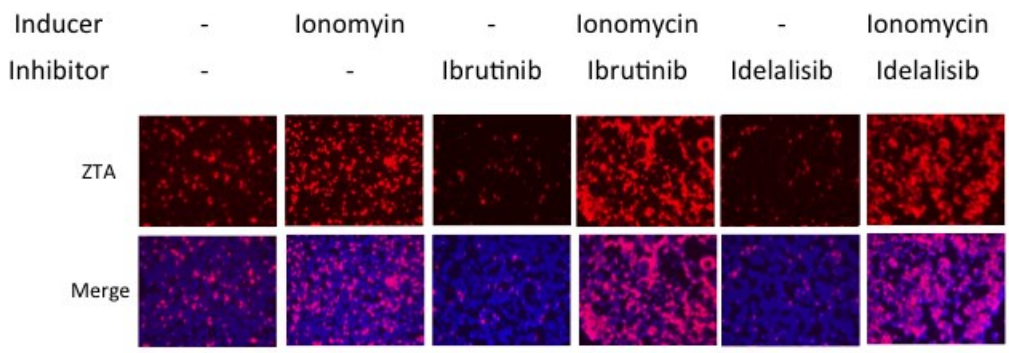

Figure 1-8. Ibrutinib and idelalisib do not block ionomycin-induced EBV. Immunofluorescence staining showing ZTA. BX1-Akata cells were pre-treated with the indicated kinase inhibitors and then treated with ionomycin after 1 hour. Cells were treated with 1 μM ionomycin.

Figure 1-9.

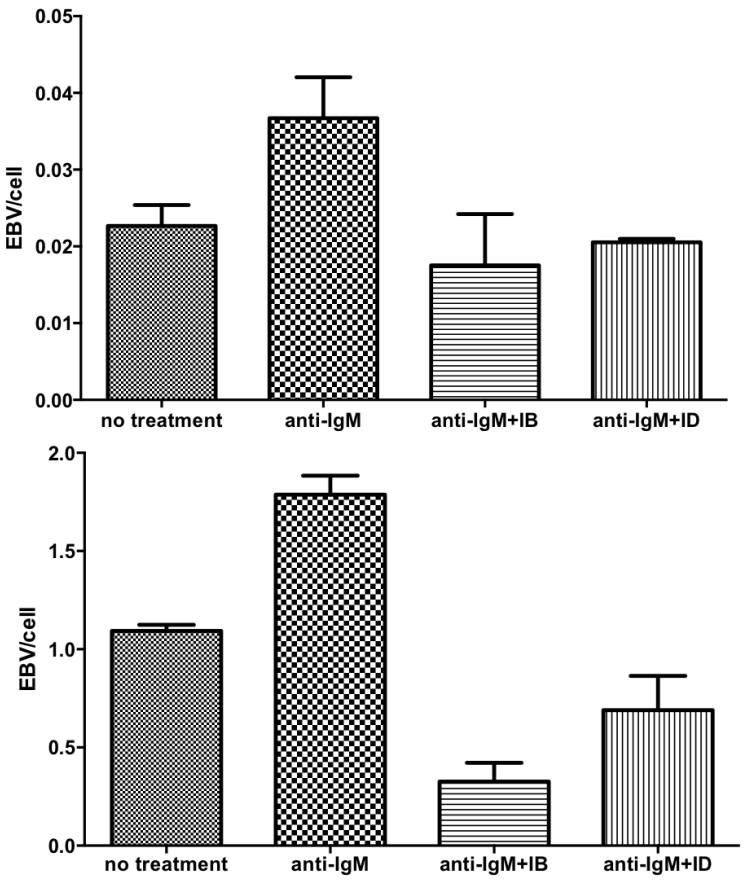

Figure 1-9.

BCR-mediated EBV activation in naturally infected EBV-positive B cells. EBV reactivation is inhibited by ibrutinib and idelalisib. Peripheral blood mononuclear cells were obtained from two patients with high EBV copy number, but no malignancy. B cells were negatively selected using magnetic-activated cell sorting. Treatment with anti-IgM led to increased EBV genome amplification at 48 hours. Pretreatment with 1μM ibrutinib or idelalisib 1 hour before anti-IgM treatment prevented EBV amplification.

Discussion

This work demonstrates that drugs targeting the BCR pathway block BCR-mediated EBV activation in EBV-positive Burkitt lymphoma cell lines. We were also able to show this effect in naturally infected B cells. Thus this phenomenon may be applicable *in vivo* as well.

Ibrutinib, idelalisib and dasatinib are used in the treatment of various chronic B cell cancers (86, 87). The BCR effects of dasatinib are off-target, and while this drug is used to inhibit BCR-ABL, the off-target effect is potent enough to enable BCR pathway inhibition. These agents are typically administered until there is tumor progression, and patients may be treated with these compounds for years.

It is possible that the BTK and PI3Kδ inhibitors will have an effect on the long-term EBV reservoir and EBV. If maintenance of that EBV reservoir requires periodic infection of previously uninfected cells, then inhibiting EBV lytic production might alter the ability to maintain that EBV reservoir. Inhibiting lytic replication and subsequent infection of new cells might result in fewer EBV-positive cells and reduced EBV malignancy. Alternatively, if periodic EBV activation results in the death of virus harboring cells that might evolve to cancerous cells, then blocking lytic activation might increase EBV malignancy.

The results seen here suggest that stimulation of the BCR pathway leads to EBV activation in freshly isolated peripheral blood B cells *in vitro*. Furthermore, drugs that block important kinases of the BCR pathway blocked EBV activation *in vitro* and in freshly isolated B cells. As these drugs are more commonly used and

typically are treated over long periods of time, further studies of their effects on EBV-associated phenomena are worth being undertaken. Inhibitors of BTK, such as ibrutinib, are widely used in treating chronic lymphocytic leukemia. It has been suggested that EBV copy number may have prognostic significance, and EBV-related complications are well recognized. Thus the impact of these agents in chronic lymphocytic leukemia would seem to be an appropriate focus of future research.

Chapter 2: Characterization of immunosuppressants on B cell receptor-mediated activation of Epstein-Barr virus

Summary

The immunosuppressive drug rapamycin blocks a component of the B cell receptor pathway, mTORC1. Therefore, one might expect rapamycin to block BCR-mediated lytic induction of EBV. While the immunosuppressive drugs cyclosporine and tacrolimus inhibit BCR-driven activation of lytic infection, rapamycin, also an immunosuppressive drug, does not. Rapamycin also binds to FKBP12. Non-immunosuppressive analogs of tacrolimus, which bind FKBP12 but not calcineurin, did not block anti-Ig-mediated EBV activation. Torin2, an mTORC1 and mTORC2 inhibitor blocks anti-Ig-mediated activation of EBV, suggesting that mTORC2 contributes to the signaling cascade from the B cell receptor that results in the activation of EBV. Finally, the difference between the effects of calcineurin inhibitors and rapamycin on EBV reactivation following BCR pathway stimulation suggests the possibility that supplanting treatment by calcineurin inhibitors with rapamycin could result in a lower incidence of post-transplant lymphoproliferative diseases.

Introduction

Calcineurin inhibitors and rapamycin are both inhibitors of T cell function and are used in transplantation to prevent organ rejection in solid organ transplantation or graft versus host disease in hematopoietic cell transplantation. In some cases, EBV-driven B cell malignancies can arise as a result of loss of immunosurveillance of EBV-transformed cells. As was previously shown (53), calcineurin inhibitors block EBV activation following BCR stimulation. A recent study found that in renal transplant recipients who were EBV-seronegative prior to transplant, treatment with rapamycin and mycophenolate was associated with a lower risk of post-transplant lymphoproliferative disease than tacrolimus or mycophenolate (88).

In the previous chapter, modulators of the B cell receptor pathway resulted in the blocking of anti-Ig-mediated activation of EBV. The immunosuppressive rapamycin also blocks a component (albeit farther downstream) of the B cell receptor pathway, mTORC1. Rapamycin also binds to FKBP12, a binding domain it shares with tacrolimus. We therefore set out to determine if rapamycin had an effect on BCR-stimulated EBV activation.

Materials and Methods

Reagents and Antibodies. Antibodies against phospho-AKT (Ser473) (Cat# 9271L), were obtained from Cell Signaling Technologies. Antibodies against EBV ZTA (Cat# sc-53904) were obtained from Santa Cruz Biotechnology and against β-actin (Cat# A5441) from Sigma-Aldrich. Anti-IgG was purchased from Sigma-Aldrich (I5260). Antibodies against phospho-S6K (Thr389) (Cat# 56311) were purchased from Sigma-Aldrich. Antibodies against phospho mTOR (Ser2448) (Cat# 2971S) and mTOR (Cat# 2972S) were purchased from Cell Signaling Technologies. Rapamycin (Cat# 37094), Torin2 (Cat# SML1224) and tacrolimus (Cat# F4679) were purchased from Sigma-Aldrich. In all treatments, anti-IgG was used at 10 μg/mL. Secondary anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch. Ibrutinib was purchased from ApexBio. Idelalisib and dasatinib were obtained from MedKoo. Ionomycin was purchased from Sigma-Aldrich. Non-immunosuppressive FK506 analogues were synthesized and characterized as described below.

Cell Lines and Culture. An engineered Akata cell line derivative (BX1 Akata) that carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP) was a gift from L. Hutt-Fletcher (80). BX1 Akata cells were cultured in RPMI 1640 media (Mediatech) with 10% FBS (Mediatech), 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies), 100 mM L-glutamine (Life Technologies), and 500 μg/mL G418. Jurkat cells were cultured in RPMI 1640 media (Mediatech) with 10% FBS (Mediatech), 100 units/mL

penicillin, 100 µg/mL streptomycin (Life Technologies), and 100 mM L-glutamine (Life Technologies).

Fluorescence Microscopy. $1.5x10^5$ cells were taken from culture and washed with PBS. Cells were then spun onto microscope slides using a Cytospin centrifuge. Cells were fixed and permeabilized with ice-cold methanol for 15 minutes and blocked in 5% BSA in PBS for 30 minutes at room temperature. ZTA antibodies (Santa Cruz) were diluted 1:50 in 5% BSA in PBS and applied to the cells for 1 hour at room temperature. The attached cells were then washed 3 times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS. Cy3 goat anti-mouse antibodies (Jackson Immunoresearch) were diluted 1:50 in 5% BSA in PBS and applied to the cells for 1h at room temperature in the dark. The slides were then washed 3 times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS. The cells were stained with Vectashield mounting media with DAPI purchased from Vector Laboratories. For GFP, BX-1 Akata cells were cultured as above and treated as indicated in the figures. A ZOE Fluorescent Cell Imager (Bio-Rad) was used to image the fixed cells for Cy3 and live cells for GFP.

Quantitative PCR. DNA was isolated from cells using the QIAamp DNA isolation kit from Qiagen. Quantitative PCR assay of the Bam W repeat region of the EBV genome was performed for measuring viral load. A BamW probe (Integrated DNA Technologies) (5′ (FAM) CACACACTACACACACCCACCCGTCTC (BH1) 3′) was used in conjunction with SsoAdvance Supermix (Bio-Rad). BamW primers were obtained from Integrated DNA Technologies (fwd 5′ CCCAACACTCCACCACACC 3’ and rev 5′

TCTTAGGAGCTGTCCGAGGG 3′). Copy number was determined by comparing to a serial dilution of the Namalwa cell line. The concentration of primers was 500 nM. The probe concentration was 200 nM. 2 µL DNA at 50 ng/mL was used per reaction. Reaction size was 20 µL. The reaction was set at 95°C for 2 minutes for 1 cycle and then 95°C for 5 seconds and 60°C for 10 seconds for a total of 40 cycles on a CFX96 real time thermocycler from BioRad. For viral load quantification by qPCR, cells were treated with ibrutinib, idelalisib or dasatinib for 1h prior to treatment with anti-IgG, and DNA was isolated after 48h. For Zta mRNA quantification, cells were treated as above and RNA was isolated after 24 hours (RNEasy kit from Qiagen). cDNA was then made from the RNA using the iScript reverse synthase kit from BioRad. cDNA volume corresponding to 25 ng total RNA was amplified using the SsoFast Evagreen kit from BioRad on a CFX96 real time thermocycler from BioRad. The cDNA was amplified using a PCR program of 95°C for 30 seconds once and then 95°C for 5 seconds, and 6°C for 5 seconds for 40 cycles. Primers used for Zta were fwd 5'ACATCTGCTTCAACAGGAGG5 3' and rev 5'AGCAGACATTGGTGTTCCAC 3'.

Immunoblots. For phospho-protein immunoblots, cells were serum-starved for three hours and pre-treated with ibrutinib, idelalisib or dasatinib for 1h prior to treatment by anti-IgG. Protein was isolated ten minutes after treatment by anti-IgG. For ZTA blots, cells were treated with ibrutinib, idelalisib or dasatinib for 1 hour prior to anti-IgG treatment and protein was extracted 24h after anti-IgG treatment. To prepare protein extracts, 1.5 X 10^7 cells were pelleted by

centrifugation and washed in PBS. The pellet was resuspended in a 10 mM HEPES, pH 7.9, 10 mM KCl, 100 μM EDTA and 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies) buffer and incubated on ice for 15 min. NP-40 was added to a final concentration of 0.6% and the cells were lysed by brief vortexing. The cytosolic supernatant was separated from the nuclear pellet by centrifugation at 10,000 rpm for 30s at 4°C. The nuclear pellet was then resuspended in a 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA buffer with 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies) followed by rotation for 15 minutes at 4°C. The nuclear supernatant was isolated by centrifugation at 13,000 rpm for 5 minutes at 4°C. Equal amounts of protein per sample were separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes using an iBLOT transfer device (Life Technologies). After probing with primary antibodies, horseradish peroxidase-conjugated antibodies (GE) were applied against the primary antibodies. The immunoblots were imaged using Amersham ECL-Prime chemiluminescent reagent (GE Health). The film used was HyBlot CL autoradiography film (Denville Scientific).

Luciferase reporter assay. NFAT luciferase reporter assay was performed as described previously (89). Briefly, Jurkat T cells transfected with an NFAT reporter construct were seeded into 96 well plates at a density of $5x10^4$/180μL/well. Drugs were serially diluted in DMSO as 1000X stocks, which were subsequently diluted to 20X in serum-free RPMI 1640 media before addition to the 96 well plates. After 30 minutes of drug incubation, 40 nM of phorbol myristate acetate (PMA) and 1 μM of ionomycin were added to stimulate

cells. After a 6-hour incubation, plates were centrifuged and the supernatant was removed. Cells were immediately lysed *in situ* by adding 100 µL/well lysis buffer and kept at room temperature for 30 minutes. The luciferase activity was determined with sequential injection of luciferin solution into each well followed by photon emission detection (Glomax reader, Promega).

Synthesis and characterization of FK506 analogs.

Compound C: To a mixture of 4-vinylbenzyl chloride (A, 0.4 g, 2.6 mmol), K_2CO_3 (1g, 7.2 mmol), KI (0.1g, 0.6 mmol) in anhydrous DMF (5 mL) under Ar was added 1-naphthol (B, 0.3g, 2 mmol). After stirring for 40h at RT, the reaction was quenched with 100 ml H_2O. The mixture was extracted with DCM, washed with brine, dried and concentrated. The resulting residue was purified by silica gel column using 10% ethyl acetate in hexane as an eluent to give compound C as a white solid. Yield: 62%. ^{1}H-NMR (500 MHz, CDCl3) δ 8.36 – 8.32 (m, 1H), 7.82-7.79 (m, 1H), 7.52 – 7.42 (m, 8H), 6.88 (d, *J* = 7.3 Hz, 1H), 6.75 (dd, *J* = 17.6, 11.0 Hz, 1H), 5.78 (dd, *J* = 17.5, 0.8 Hz, 1H), 5.27 (dd, *J*=11.0, 0.7 Hz, 1H), 5.24 (s, 2H).

FKN4: To a solution of FK506 (D, 10 mg, 0.012 mmol) and compound C (15.6 mg, 0.187 mmol) in anhydrous DCE (3 mL), 10 mol% Hoveyda-Grubbs 2nd generation catalyst in anhydrous DCE (1mL) was added at rt (90, 91). The reaction was irradiated under microwave for 5 minutes at 140^{o}C with shaking. The mixture was filtered and thoroughly washed with DCM (5×3ml). The filtrate was collected, concentrated, and resuspended in DMSO for LC-MS analysis and purification. Yield: 30%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.31 – 8.25 (m, 1H), 7.77 –

7.72 (m, 1H), 7.46 – 7.35 (m, 8H), 6.82 (d, *J*=7.3Hz, 1H), 6.36 (d, J=15.5Hz, 1H), 6.13-6.01 (m, 1H), 5.27(s, 1H), 5.17 (s, 2H), 5.07-4.97 (m, 3H), 4.60-4.56 (m, 1H), 4.37 (d, J=14Hz, 1H), 4.20 (s, 1H), 3.95-3.77 (m, 2H), 3.65-3.60 (m, 1H), 3.55-3.47 (m, 1H), 3.37-3.29 (m, 13H), 2.99-2.90 (m, 2H), 2.79-2.70 (m, 1H), 2.64-2.50 (m, 2H), 2.32-2.18 (m, 4H), 2.06-2.0(m, 2H), 1.78-1.65 (m, 5H), 1.65-1.54 (m, 14H), 0.94-0.84 (m, 9H), 0.84-0.80 (m, 3H). Mass spectrometry: found m/z=1059.1. $[M + Na]^+$, calculated 1036.3.

FKAM**:** To a solution of FK506 (100 mg, 0.120 mmol) and 30 mol% Zhan1b ruthenium catalyst in 3 mL anhydrous DCE, 4-allylmorpholine (18.1 µL, 0.132 mmol) was added. The mixture was stirred for 30 sec before microwave irradiation at 120°C for 20 mins. The crude product was then purified using flash chromatography (0-25% gradient MeOH in DCM), followed by preparative-TLC (9:1 DCM:MeOH). Yield: 25%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.51 (s, 2H), 5.33 (s, 1H), 5.19 (s, 1H) 5.12-4.98 (m, 2H), 4.60-4.40 (m, 1H), 3.98-3.85 (m, 1H), 3.75-3.65 (d, 5H), 3.61-3.56 (d, 1H), 3.42-3.32 (m, 8H), 3.3 (s, 2H), 3.05-2.89 (m, 3H), 2.76-2.65 (m, 2H), 2.43 (s,5H), 2.35-2.25 (m, 2H), 2.20-2.12 (d, 2H), 2.10-1.93 (dd, 4H), 1.89 (s, 1H), 1.79-1.71 (m, 2H), 1.70-1.55 (m, 7H), 1.51-1.41 (m, 2H), 1.34-1.16 (m, 11H), 1.08-1.12 (d, 2H), 1.12-0.97 (d, 2H), 0.96-0.79(dt, 9H). High-resolution mass spectrometry: found m/z = 903.5614. $[M + H]^+$, calculated 903.5582.

Results

It has been previously reported that anti-Ig induction of the EBV lytic cycle in Akata cells can be blocked by the immunosuppressant drugs cyclosporin A and tacrolimus (53). As shown in Fig. 2-1a, we first confirmed the observation that cyclosporine A and tacrolimus block anti-IgG-induced EBV lytic activation. It appears that rapamycin weakly activates lytic infection (Fig. 2-2b). This may reflect activation of a feedback loop between mTORC1 and AKT.

Previous investigations reported that rapamycin inhibited EBV lytic activation, albeit in different cell types and in conjunction with other lytic inducers (92). However, rapamycin, does not block anti-Ig-induced EBV activation (Fig. 2-1c and 2-2c). The investigators who had reported inhibition of lytic EBV infection with rapamycin had not studied anti-Ig induced EBV activation but instead had studied NaB and TPA EBV activation. This led us to investigate differential effects of immunosuppressants on EBV lytic activation by various inducers. We pretreated cells with cyclosporine A, tacrolimus or rapamycin followed by treatment with anti-IgG, NaB, TPA or ionomycin. We confirmed the previous report that rapamycin blocked NaB and TPA-induced EBV lytic activation (Fig. 2-1e) and found that cyclosporine A and tacrolimus did not (data not shown). In contrast, while cyclosporine A and tacrolimus block ionomycin-induced EBV lytic activation, rapamycin does not (Fig. 2-1d, 2-2d). These results suggest that cyclosporine A, tacrolimus and rapamycin, although all immunosuppressive agents, have very different effects on EBV activation.

Tacrolimus and rapamycin both form binary complexes with FKBP (93). The FKBP-tacrolimus complex binds to and inhibits the enzymatic activity of the protein phosphatase calcineurin, blocking calcium-dependent intracellular signaling, while the FKBP-rapamycin complex binds to the protein kinase mTOR. Tacrolimus and rapamycin share a nearly identical FKBP-binding domain (FKBD) but each possesses distinct effector domains responsible for interactions with calcineurin and mTOR, respectively. The failure of rapamycin to inhibit Ig-induced lytic activation suggested that binding FKBP is not sufficient to block EBV lytic activation. To further verify this hypothesis, we synthesized two non-immunosuppressive analogs of tacrolimus and investigated their effects on EBV lytic activation by anti-IgG (Fig. 2-3). The two analogues, FKAM and FKN4, share the same FKBP-binding domain, but have altered effector domains. Thus, new substitutions were added to the allyl group of FK506 to generate FKN4 and FKAM, respectively (Fig. 2-4). The presence of the newly added 'bumps' in the effector domain of FK506 is expected to prevent FKN4 and FKAM from binding to calcineurin without affecting their interactions with FKBP. The lack of inhibition of calcineurin was verified using an NFAT-luciferase reporter gene, which upon transfection into Jurkat T cells, can be stimulated by PMA and ionomycin. While FK506 potently inhibited the NFAT-luciferase reporter, neither FKN4 nor FNAM had an appreciable effect on the reporter activity at the highest concentrations tested (Fig. 2-5). The interactions of FKN4 and FKAM with FKBP are demonstrated by their ability to compete with FK506 and block inhibition of NFAT-luciferase (Fig. 2-6). Next, we determined the effects of the non-

immunosuppressive analogs on EBV lytic activation in response to anti-IgG stimulation. BX1-Akata cells were pre-treated with these analogs at varying doses for 1 hour and then induced with anti-IgG. EBV activation was assessed by GFP expression 24 hours post-treatment. Neither FKN4 nor FKAM affected GFP expression induced by anti-IgG, further supporting the notion that FKBP-binding is not sufficient to block EBV lytic activation and inhibitory effects observed for cyclosporine A and tacrolimus were mediated through inhibition of calcineurin (Fig. 2-7).

To further investigate the impact of blocking mTOR signaling on EBV lytic activation, we used the mTOR active site inhibitor torin2. While rapamycin inhibits mTOR complex1 (mTORC1), torin2 has been shown to block both mTORC1 and mTORC2 (94). BX1-Akata cells were pre-treated with either rapamycin or torin2 and induced with anti-IgG after 30 minutes. ZTA expression was assessed by immunofluorescence and immunoblot, while Zta RNA level was assessed by qRT-PCR 24 hours after the treatment (Fig. 2-8 and 2-9). Rapamycin did not block anti-IgG induced EBV lytic activation, but torin2 did. Treatment by anti-IgG increased phosphorylation of mTOR, AKT and S6K, a downstream target of mTOR kinase. We found that both rapamycin and torin2 blocked phosphorylation of mTOR as well as its downstream target, S6K (Fig. 2-10). However, profound inhibition of AKT phosphorylation was shown after 30 minutes in torin2 treated cells, as it blocks both mTORC1 and mTORC2, which has a positive feedback loop with AKT. Rapamycin also blocked phosphorylation of AKT, but to a lesser degree. Torin2 treatment alone also resulted in a

decrease in ZTA expression, likely due to inhibition of basal BCR signaling (Fig. 2-1). These results suggest that mTORC2 and likely AKT activity is crucial for anti-igG mediated EBV lytic activation in the Akata cell line.

Figure 2-1

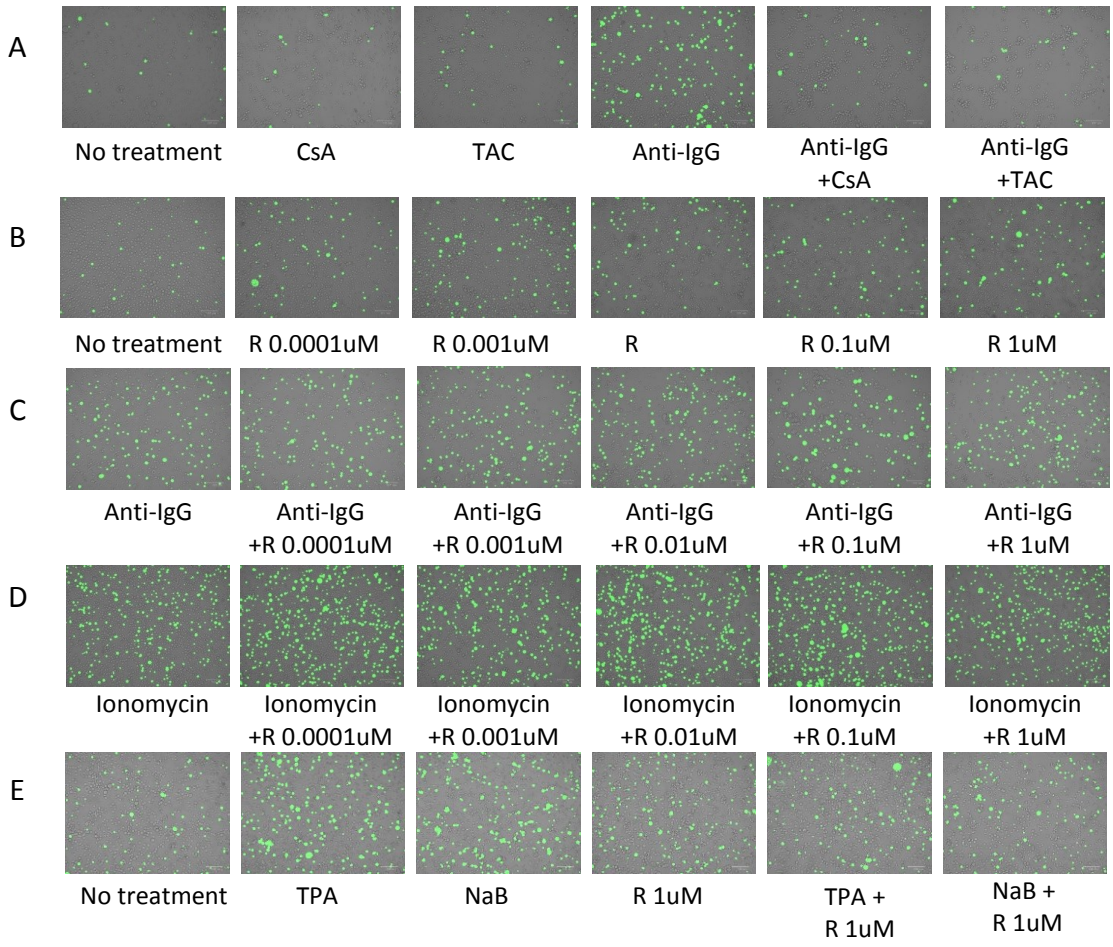

Figure 2-1. **Rapamycin does not block anti-IgG induced lytic activation.** A. BX1-Akata cells were pre-treated with 1 μM CsA or 10 nM TAC for 1 hour, followed by treatment with anti-IgG. B. BX1-Akata cells were treated with various doses of rapamycin (R). C and D. BX1-Akata cells were pre-treated with rapamycin using varying doses for 1 hour and followed by induction with anti-IgG (C) or ionomycin (D). E. BX1-Akata cells were treated with rapamycin for 1 hour, followed by treatment with NaB or TPA. Cells were treated with 1 μM ionomycin, 20 ng/mL TPA or 3 mM NaB unless otherwise indicated.

Figure 2-2.

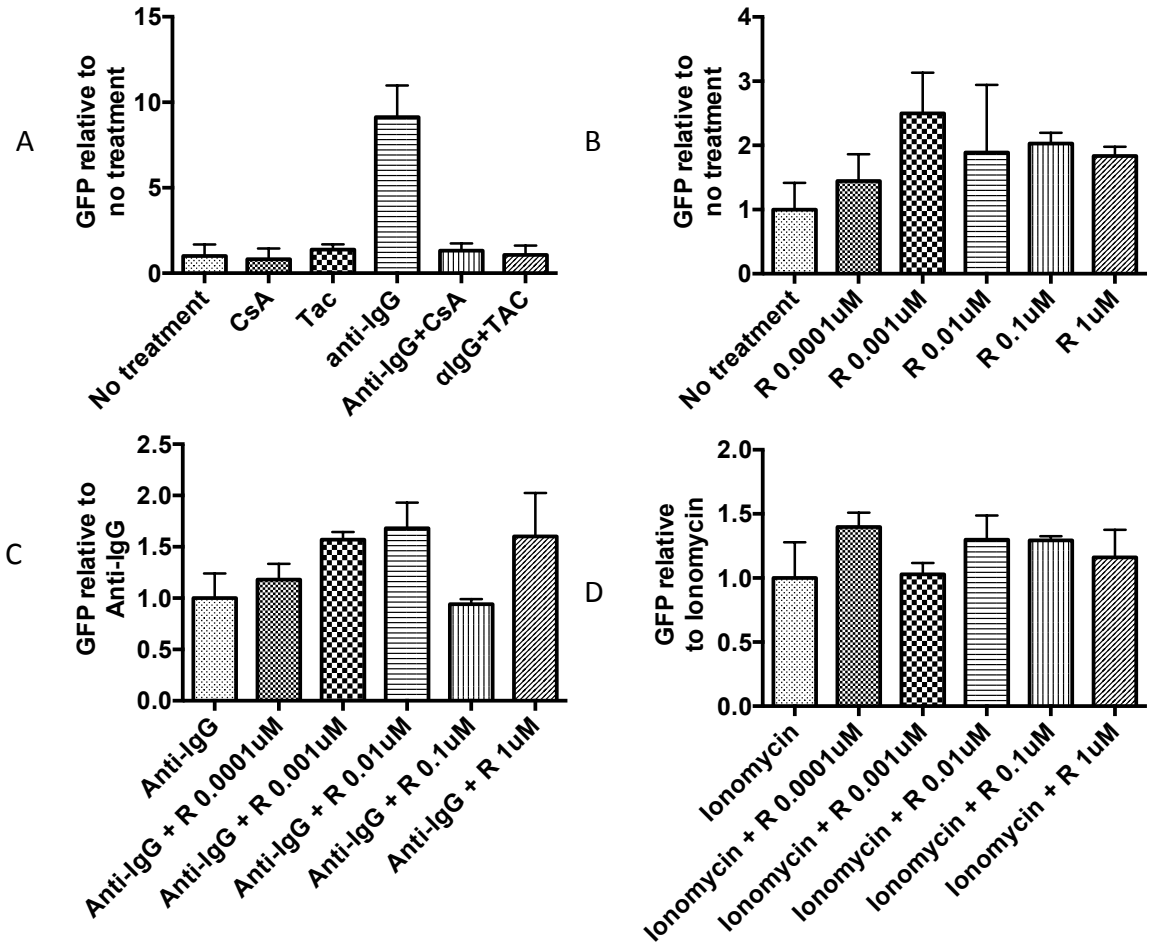

Figure 2-2. Rapamycin does not block BCR-mediated or calcium ionphore-mediated lytic EBV. Fluorescence microscopy was used to determine the number of GFP-positive cells. GFP-positive cells were quantified and values were graphed as a function of the experimental control. A, B, C, and D are quantification of Figure 2-1 A, B, C and D, respectively. Cells were treated with 1 μM ionomycin, 20 ng/mL TPA or 3 mM NaB unless otherwise indicated.

Figure 2-3.

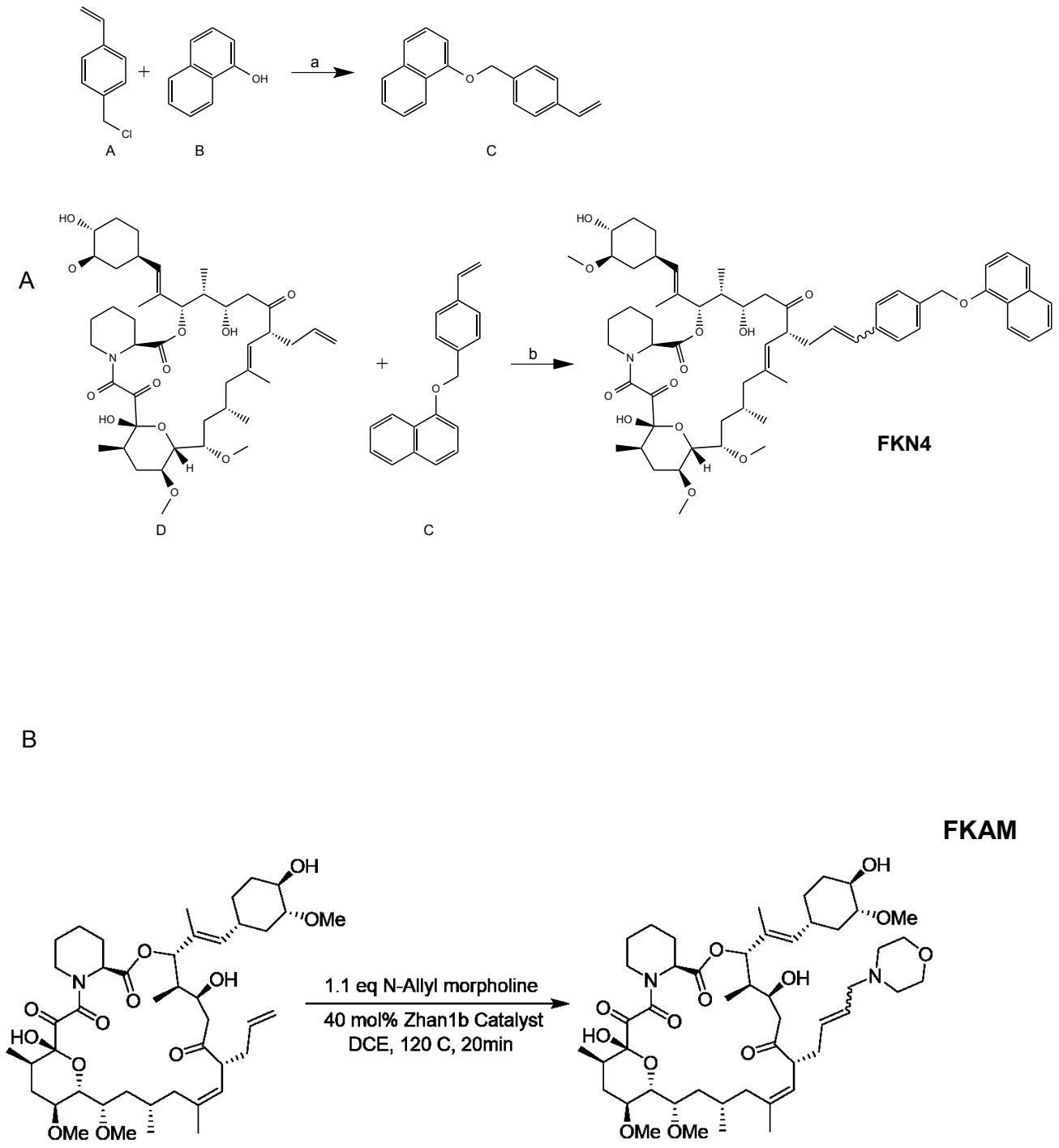

Figure 2-3. Synthesis of tacrolimus analogs. A. Synthetic scheme of FKN4. B. Synthetic scheme of FKAM.

Figure 2-4.

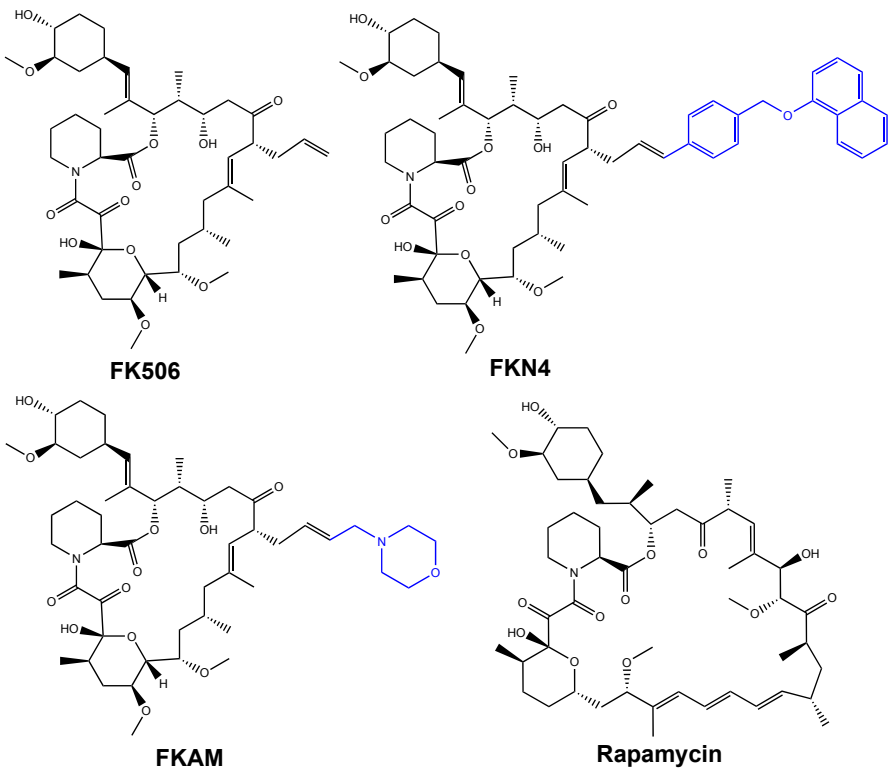

Figure 2-4.

Structures of FK506, rapamycin and non-immunosuppressive FK506 analogs FKN4 and FKAM. The newly added substitutions in FKN4 and FKAM are colored blue.

Figure 2-5.

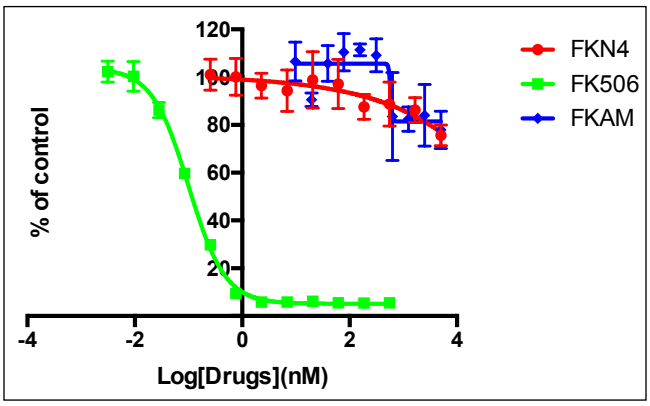

Figure 2-5. Effects of FK506, FKN4 and FKAM on the activation of an NFAT-luciferase reporter gene stimulated with PMA and ionomycin in Jurkat T cells. After 30 minutes of drug incubation, 40 nM of phorbol myristate acetate (PMA) and 1 μM of ionomycin were added to stimulate cells. After a 6-hour incubation, cells were lysed and kept at room temperature for 30 minutes, and luciferase activity was measured.

Figure 2-6.

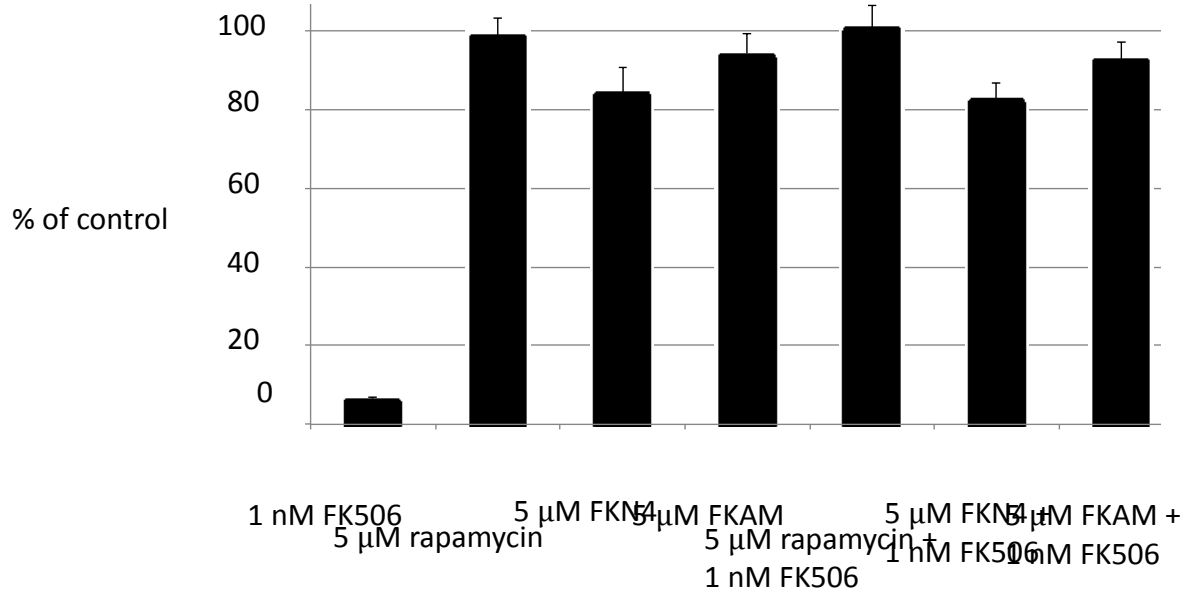

Figure 2-6. FK506 analogs and rapamycin reverse FK506 effects in an NFAT luciferase reporter gene competition assay in stimulated Jurkat T cells.

Effects of FK506, FKN4, FKAM and rapamycin on the activation of an NFAT-luciferase reporter gene stimulated with PMA and ionomycin in Jurkat T cells. FKN4, FKAM and rapamycin were then treated in the presence of FK506.

Figure 2-7.

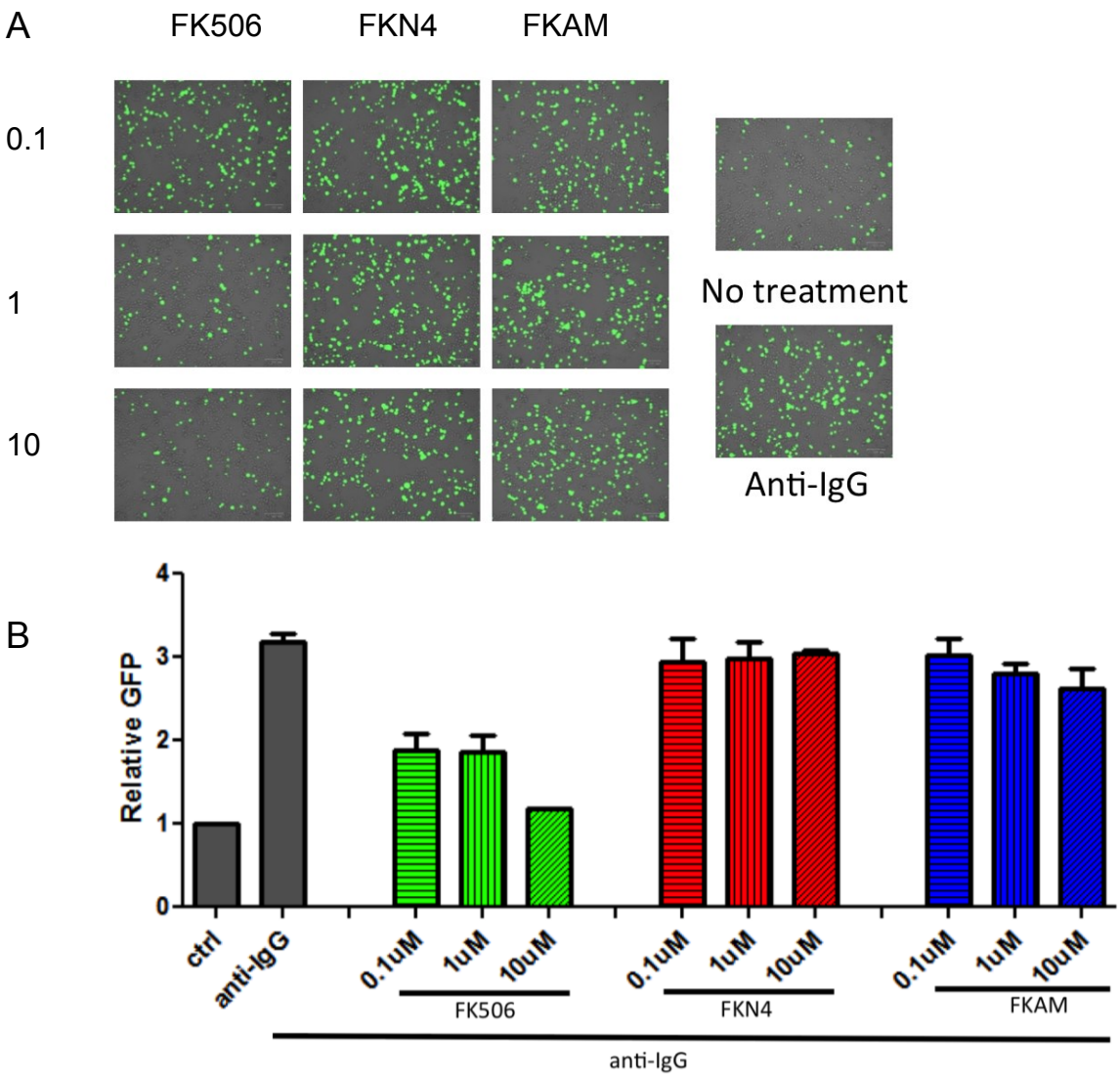

Figure 2-7. Nonimmunosuppressive FK506 analogs do not block BCR-mediated induction of EBV. BX1-Akata cells were pre-treated with tacrolimus and tacrolimus analogs FKAM and FKN4 using varying doses for 1 hour followed by induction with anti-IgG for 24 hours. A. Fluorescent cells were imaged for GFP. B. GFP-positive cells were counted and compared with an untreated sample.

Figure 2-8.

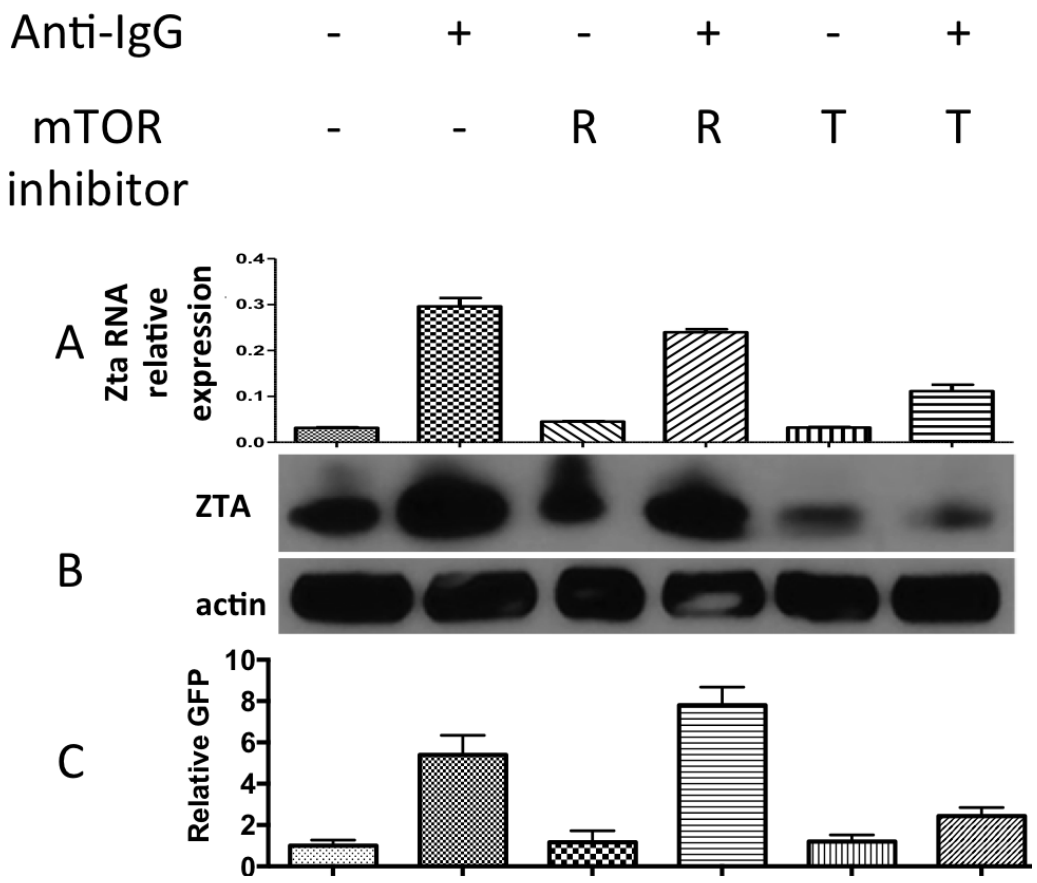

Figure 2-8. mTORC2 activity is crucial for B cell receptor (BCR)-mediated EBV lytic activation. BX1-Akata cells were pre-treated with rapamycin (R) or torin2 (T) for 30 minutes followed by induction with anti-IgG. A. Zta RNA level was measured by qRT-PCR 24 hours after either rapamycin or torin2 treatment and normalized to GAPDH. B. ZTA protein level was assessed by immunoblot 24 hours after treatment. C. GFP-positive cells were counted and compared with an untreated sample.

Figure 2-9.

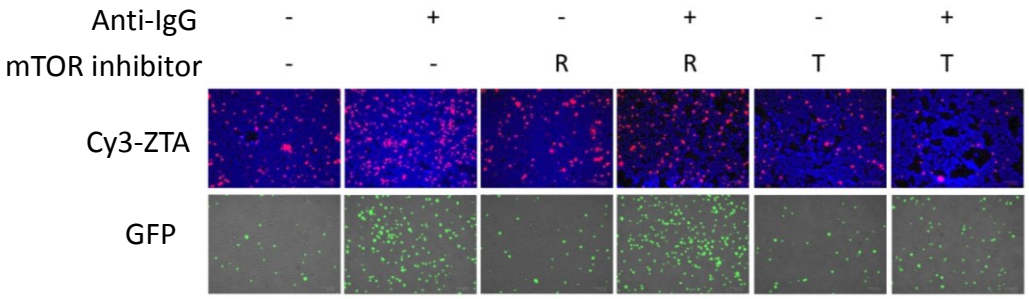

Figure 2-9. Torin2, but not rapamycin, blocks lytic induction of EBV. BX-1 Akata cells were pretreated with the mTORC1 inhibitor rapamycin and the mTORC1 and mTORC2 inhibitor torin2 for 30 minutes prior to treatment with anti-IgG. Top panel: Immunofluorescence showing ZTA induction. Bottom panel: GFP.

Figure 2-10.

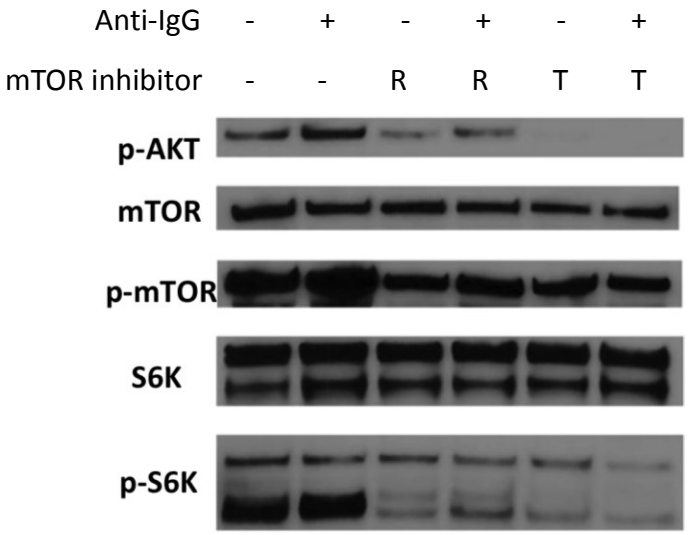

Figure 2-10. Torin2, but not rapamycin, blocks phosphorylation of mTOR and S6K. BX-1 Akata cells were pretreated with the mTORC1 inhibitor rapamycin and the mTORC1 and mTORC2 inhibitor torin2 for 30 minutes prior to treatment with anti-IgG. Phosphorylation of AKT, mTOR and S6K were assessed by immunoblot 10 minutes after anti-IgG treatment.

Discussion

Immunosuppressive treatments have been shown to play a central role in pathogenesis of EBV-associated lymphoproliferative diseases (95). The onset of disease is believed to be due to the effects of immunosuppressives on T cell function and loss of immune system control of EBV-driven B-cell lymphoproliferation (96). More recently, rapamycin has replaced or been used in combination with calcineurin inhibitors for therapy in many transplantation regimens. Studies have suggested that while calcineurin inhibitors block T cell function, in some cases, rapamycin has the opposite effect (72).

While calcineurin inhibitors and rapamycin are both used as immunosuppressants in transplant regimens and both block T cell function, they have opposite effects on BCR-mediated EBV activation. As previously reported (53) and confirmed here, tacrolimus and cyclosporine inhibit BCR-mediated EBV activation. Interestingly, we found that rapamycin did not block such activation. Therefore, in the setting of post-transplant lymphoproliferative disease, these drugs may have different outcomes. There is a possibility that EBV-driven malignancies, which arise under a treatment regimen of calcineurin inhibitors, may be less viable in the presence of rapamycin alone.

The differences in post-transplant lymphoproliferative disease between rapamycin and calcineurin inhibitors indicate that further work analyzing the role of BCR-mediated EBV activation may prove useful in uncovering causative agents of such diseases.

OVERALL DISCUSSION

The studies presented here demonstrate that pharmacologic agents targeting the BCR pathway block BCR-mediated activation of the EBV lytic cycle. Furthermore, with the investigation of unmanipulated, naturally infected B cells isolated from human host, we have presented evidence that BCR activation may be relevant *in vivo* as well.

Since the early days of organ transplantation, pharmacologic agents have been recognized to play an important role in the pathogenesis of EBV-associated lymphoproliferative diseases (95). Immunosuppressive agents such as azathioprine, cyclosporine, tacrolimus, mycophenolate, anti-thymocyte globulin, OKT3 and others have been associated with an increased risk of post-transplant lymphoproliferative disease. The increased risk was generally attributed to drug effects on T cell function and resultant loss of control of EBV-driven B-cell lymphoproliferation (96). In more recent years, rapamycin has often replaced or supplemented calcineurin inhibitors in many transplantation regimens. Evidence has been presented that demonstrate the calcineurin inhibitors block T cell function, whereas in some special instances rapamycin enhances T cell function (72). For example, in a genetic immunodeficiency syndrome associated with activation of PI3Kδ, rapamycin has shown promise as a therapeutic because it enhances antiviral T cell function (97). Similarly, rapamycin may correct the antiviral deficiency associated with belatacept, a second-generation CTLA4-Ig derivative used in organ transplantation (72).

In this report, our focus is not on T cells but on B cells (98). With regard to B cells, it has previously been reported that cyclosporine and tarcrolimus increase the viability of spontaneous EBV-lymphoblastoid cell lines, possibly reflecting partial protection from Fas-mediated apoptosis and this phenomenon may also occur *in vivo* and play a role in the pathogenesis of post-transplant lymphoproliferative disorder (99). Conversely, antibody mediated B cell depletion has long been recognized as an effective intervention for EBV-associated post-transplant lymphoproliferative disease (77, 100). The EBV latency reservoir is the resting B cell and depleting the B cell reservoir reduces both pools of infected cells and those that might become infected (101).

Little is known of the regulation of viral activation in latently infected B cells *in vivo,* but certainly BCR signaling may play a role. BCR signaling has been used as a tool in the laboratory to activate lytic infection in certain cell lines *in vitro* for many years. In this work, we show that BCR signaling also activates lytic infection in freshly isolated, naturally infected B-lymphocytes from human host. Furthermore, we show that pharmacologic agents that inhibit BCR signaling also inhibit EBV lytic activation.

These BCR inhibitors in aggregate are used in the treatment of chronic lymphocytic leukemia, mantle cell lymphoma, Waldenstrom macroglobulinemia, marginal zone lymphoma, follicular lymphoma and chronic myeloid leukemia (86, 87). We note that the BCR effects of dasatinib on BCR signaling are off target and that the agent is used to inhibit BCR-ABL in the treatment of chronic myelocytic leukemia. All of these agents are orally administered and in contrast

to earlier generations of antineoplastic are typically prescribed until there is tumor progression, i.e. patients may be treated with these agents for months or years. None of these malignancies is typically associated with EBV, although high EBV copy number in blood has been reported in some patients with chronic lymphocytic leukemia (102, 103) and chronic lymphocytic leukemia may evolve into EBV-associated diffuse large B cell lymphoma or Hodgkin lymphoma (104, 105).

We suspect that the BTK and PI3Kδ inhibitors will impact the long-term EBV reservoir and EBV viremia. However, it is difficult to predict from first principles what these effects will be. Thus, if sustenance of that reservoir required intermittent infection of previously uninfected cells, then blocking EBV activation might interfere with the ability to maintain that reservoir. Preventing lytic replication and new rounds of infection might result in fewer EBV-infected cells and less EBV malignancy. Alternatively, if periodic lytic EBV activation results in the death of virus harboring cells that might evolve to malignant cells, then blocking lytic activation might increase EBV malignancy.

Although calcineurin inhibitors and rapamycin are both inhibitors of T cell function and are both used in transplantation to suppress or prevent organ rejection in solid organ transplantation or graft versus host disease in allogeneic hematopoietic cell transplantation, these agents have markedly different effects from each other on BCR-mediated EBV activation in B cells. As was previously shown (53) and confirmed here, the calcineurin inhibitors block EBV activation following BCR stimulation, whereas such activation is not inhibited by rapamycin

in our experiments. Thus these agents may be expected to have very different effects with regard to post-transplant lymphoproliferative disease. A recent report indicated that in renal transplant recipients who were EBV-seronegative prior to transplant, treatment with rapamycin and mycophenolate was associated with a lower risk of post-transplant lymphoproliferative disease than tacrolimus and mycophenolate (88). Whether the difference in post-transplant lymphoproliferative disease reflects drug effects on T cells or B cells or both is not known.

In conclusion, our investigations suggest that stimulation of the BCR pathway leads to EBV lytic induction in freshly isolated peripheral blood B cells *in vitro*. Furthermore, inhibitors of the BCR pathway block EBV activation *in vitro* and in freshly isolated B cells from human host. Finally, in contrast to calcineurin inhibitors, rapamycin does not inhibit BCR-driven EBV activation. As these drugs are increasingly used widely and usually are used continuously for chronic disease over months or years, it seems that further investigation of their effects on EBV-associated phenomena are indicated. The BTK inhibitors are widely used in chronic lymphocytic leukemia. There is a suggestion that EBV copy number in blood may have prognostic significance, and EBV-related complications though rare are well recognized. Thus the impact of these agents in chronic lymphocytic leukemia would seem to be an appropriate focus of future research. Similarly, with differences in incidence of EBV-associated post-transplant lymphoproliferative disease associated with regimens that include calcineurin inhibitors, further investigation of the possible role of BCR-mediated

EBV activation in the pathogenesis of post-transplant lymphoproliferative disease may be warranted.

Future Directions

We are interested in the possibility that by pharmacologically activating or inhibiting pathways in B cells, it will be possible to prevent or treat EBV-associated B cell malignancies. There are several barriers to easily determining what effect activating or blocking B cell pathways in the context of EBV activation has on EBV-associated B cell malignancies.

To start, it is not possible to predict the long-term effects of inhibiting B cell signaling. With regard to EBV, the inhibition of B cell signaling and thus baseline lytic activation might ultimately abolish the EBV reservoir if B cell signaling and resultant lytic infection is necessary for viral maintenance. This could manifest itself in a regression of EBV-associated malignancy. Conversely, if B cell pathway inhibitors block basal EBV activation and resultant cell death, then one might expect EBV to remain in cells, but under a latent replication program. Thus EBV might more effectively harness its transforming capabilities, if EBV is most transforming under a latent replication regime. This might result in an increase in EBV-associated malignancies among those on a regimen of B cell pathway inhibitors for years.

In the case of genetic disorders affecting B cell signaling, one might imagine an activating mutation in a component of the BCR pathway resulting in increased EBV titer in a patient's blood. A recent study on activating mutations of PI3Kδ found that two out of nine patients had EBV-associated lymphoma (97). This information, when viewed in the light of how readily idelalisib, a PI3Kδ inhibitor, blocks BCR-mediated lytic activation, hints at the possibility of BCR

pathway modulation as a way to treat EBV-driven B cell malignancies. Therefore, a greater understanding of how drugs that affect the B cell pathway affect EBV activation may provide useful information to guide new approaches to patient care.

Other pharmacologic approaches to inhibiting EBV functions have been investigated, including the inhibition of EBNA1, an EBV protein present in all latently infected. This protein is responsible for EBV genome copying and partitioning during latent replication. A group of investigators showed that by adding a peptide inhibitor of EBNA1 dimerization, they could force the loss of the EBV episome in daughter cells (106, 107). The peptide had dual functionality, not only the inhibition of EBNA1 dimerization but also was fluorescent, which readily enabled its visualization in tissue sections. Targeting episomal maintenance is a promising tool for treatment in cells that are affected by drugs that block lytic activation. Thus an effect similar to depleting the viral reservoir by long-term inhibition of BCR signaling might be achieved using different strategies.

LIST OF ABBREVIATIONS

AIDS – acquired immunodeficiency syndrome

BCR – B cell receptor

BL – Burkitt lymphpma

BSA – bovine serum albumin

CsA – cyclosporine A

CTLA4 – cytotoxic T-lymphocyte-associated protein 4

DCE – 1,2-dichloroethane

DCM – dichloromethane

DMF – dimethylformaldehyde

DMSO – dimethyl sulfoxide

EBER – Epstein-barr virus-encoded small RNAs

EBNA – Epstein-Barr nuclear antigen

EBV – Epstein-Barr virus

EDTA – ethylenediaminetetraacetic acid

FKAM – tacrolimus analog

FKBP – FK506 binding protein

FKN4 – tacrolimus analog

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFP – green fluorescent protiein

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA – human leukocyte antigen

Ig – immunoglobulin

LCL – lymphoblastoid cell line

LMP – latent membrane protein

MACS – magnetic-activated cell sorting

mTORC1 – mTOR complex 1

mTORC2 – mTOR complex 2

NaB – sodium butyrate

NFAT – nuclear factor of activated T cells

OKT3 – muromonab-CD3

PBMC – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PMA – phorbol 12-myristate 13-acetate

PTLD – post-transplant lymphoproliferative disorder

SDS-PAGE – sodium dodecyl sulfide polyacrylamide gel electrophoresis

TAC – tacrolimus

TPA – 12-o-tetradecanoylphorbol-13-acetate

ACKNOWLEDGEMENTS

We thank Lindsey Hutt-Fletcher for cell lines. Funding to support this work was from R21CA188824 (RFA), R21AI101377 (SDH), the Flight Attendant Medical Research Institute (JOL) and the Johns Hopkins Institute for Clinical and Translational Research, which is funded in part by Grant UL1 TR 001079 from the National Center for Advancing Translational Sciences (NCATS).

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EDUCATION

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Fluoride as a Probe for Hydrogen-bonding in the Distal Heme Pocket of Tt H-NOX

BSc, Biochemistry *2011*

State University of New York at Stony Brook, Stony Brook

TECHNICAL EXPERTISE

Instrumentation

- CARY UV/VIS Spectrophotometer, CFX-96 real-time thermocycler, MJ thermocycler, Nucleofector II nucleofector device, AKTA protein purifier device, TSQ Vantage Triple Stage Quadrupole LC/MS Mass Spectrometer, nanoAcuity UPLC, Storm 860 Phosphor Gel Scanner, FACSCalibur and LSR II flow cytometers

Assays

- Hemeprotein redox potential, X-ray absorption fine structure, fluoride probe UV-VIS binding, standard PCR, qPCR, qRT-PCR, RT-PCR, immunoblot (western blot), immunofluorescence, fluorescence microscopy, FACS, oligonucleotide radiolabeling, intramolecular site-transfer, fluorescence anisotripy, O_2-reactive protein binding assay (Sw H-NOX), thin layer chromatography, dose-response experiments, mass spectroscopy, sequencing gel

Molecular Biology

- Site-directed mutagenesis, cloning, DNA isolation, IPTG-induced protein over-expression and his-tag purification, heme C13 aminolevulinic acid labeling, purification of O_2-reactive protein

Cell Techniques

- Bacterial culture (*E. coli)*, bacterial transformation, calcium phosphate transfection, PEI transfection, lentiviral production, harvesting and transduction, shRNA knockdown, HIV-*tat*-based gene inhibition, Epstein-Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV) induction, production and isolation, super-infection of B-cells by EBV, mammalian cell line gene modification, creation and maintenance (knock-in and -down), TALEN and CRISPR/Cas9 gene editing

Virology

- EBV and KSHV induction, gene expression analysis, harvesting and super-infection, lentiviral production, harvesting and transduction

Computer Software

PyMOL, Origin 7, GraphPad Prism, Excel, Word, PowerPoint, EndNote, ImageQuant, ImageJ

Publications

Lee, J, **Kosowicz JG**, Ambinder RF. *Oncotarget*. 2017, Vol. 8, (No. 31), pp: 50325-50326. Unveiling Kapose sarcoma viral antigens.

Kosowicz JG, Lee J, Peiffer B, Zuo G, Chen J, Liao G, Hayward SD, Liu J, Ambinder RF. *J. Virol. August 2017 vol. 91 no. 16 e00747-17.* Drug modulators of B cell signaling pathways and Epstein-Barr virus lytic activation.

Kosowicz JG, Lee J, Ambinder RF. *Nat. Biomed. Eng. 2017 Apr;1, 0059.* Seeing the ebb of a tumour virus.

Kosowicz JG, Boon EM. *J Inorg Biochem. 2013 Sep;126:91-5.* Insights into the distal heme pocket of H-NOX using fluoride as a probe for H-bonding interactions.

Schonhoft JD, **Kosowicz JG**, Stivers JT. *Biochemistry. 2013 Apr;16;52(15):2526-35.* DNA translocation by human uracil DNA glycosylase: role of DNA phosphate charge.

Presentations

Kosowicz JG, Lee J, Chen J, Hayward SD, Ambinder RF. *17th International Symposium on Epstein-Barr virus and associated diseases. University of Zurich, Zurich, Switzerland. August, 2016.* Poster session*, Kinase Inhibitors used in the Treatment of Chronic Lymphocytic Leukemia and Lymphoma Block Anti-Ig Lytic Induction.*

Kosowicz JG, Lee J, Liao G, Hayward SD, Ambinder RF. *15th International Conference on Malignancies in AIDS and Other Acquired Immunodeficiencies. National Institutes of Health, Bethesda, MD. October, 2015.* Poster session*, Reversine Blocks EBV Activation by Multiple Lytic Inducers.*
